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THE EFFECT OF TOXOHORMONE ON IRON METABOLISM

TETSUO ONO, MOCHIHIKO OHASHI and NAGASUMI YAGO
(Cancer Institute, Japanese Foundation for Cancer Research, Tokyo)

Fukuoka and Nakahara postulated that the role of toxohormone in depressing liver catalase activity may be to disturb the iron metabolism, based on their finding that either the feeding of dried liver, blood powder or iron or the simultaneous injection of iron with toxohormone were capable of counteracting the catalase depressing action *in vivo* (3). In the course of the experiments to elucidate the mechanism of catalase depression in tumor-bearing animals, the authors observed the accumulation of free protoporphyrin in the blood and liver of these animals. A porphyric state was also produced by the injection of toxohormone (8). These findings were considered to support the hypothesis that the heme synthesis in the tumor-bearing animals is impaired at the step of iron insertion into protoporphyrin rings. This hypothesis was further strengthened by the iron administration experiments, in which a large dose of iron in the diet completely prevented the porphyrin accumulation and the iron administration combined with cobalt corrected completely the amemia of tumor-bearing animals (4, 9).

Besides the catalase depression and anemia, there are other symptoms indiating the disturbance of iron metabolism in tumor-bearing animals, such as the decrease of plasma iron as well as of the liver ferritin content (10). The latter symptom was also attributed to the action of toxohormone from the malignant tumor by the experiment of Mizuhara *et al.* (6).

Recently Kampschmidt *et al.* (5) reexamined the systemic effect of toxohormone and they reported the production of anemia and decreased plasma iron level beside the depression of liver catalase and thymus involution by the administration of toxohormone from malignant tumor but not of the corresponding fraction from normal tissues. In these experiments, they emphasized the point that the plasma iron was 250–500 times more sensitive than liver catalase to the action of toxohormone and may prove of value in the test of toxohormone activity. Their findings seemed very useful in the purification of toxohormone, because more sensitive assay method than the bioassay based on the mouse liver catalase has long been looked for eagerly by the researchers on this problem. However, Kampschmidt *et al.* did not show any evidence to indicate that one and the same principle in the toxohormone preparation was responsible for the both actions, that is, depression of plasma iron and of liver catalase activity. To prove this point seemed essential before adopting the plasma

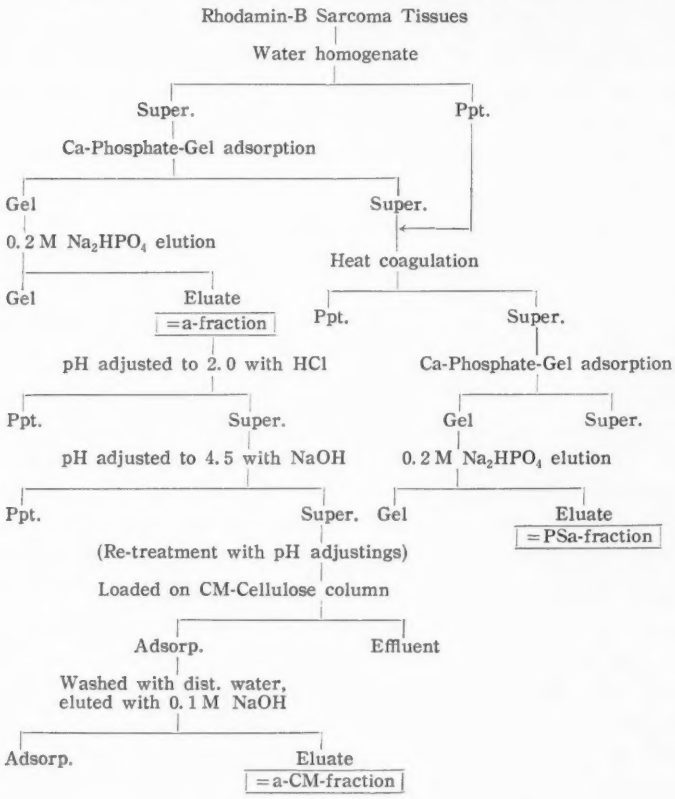
iron test as a method for the assay of toxohormone, which has been first established as catalase depressing factor *in vivo*.

In the experiments reported in this paper, we confirmed the finding of Kampschmidt *et al.* that toxohormone decreased plasma iron level of normal rat in extremely small doses. The relation between the doses to depress the plasma iron and liver catalase by 50% was established empirically. Moreover, concerning the mode of action of toxohormone in depressing plasma iron level, it was demonstrated by using ^{59}Fe-labeled plasma that the lowering of iron mobilization from the iron reserve in tissue by the action of toxohormone may be the most probable mechanism.

Material and Methods

Toxohormone Preparations: In these experiments were used four kinds of toxohormone preparations, which varied in the extracting procedures and activities, all obtained from the rat rhodamine fibrosarcoma (Umeda.) One of them, which is called

Table 1. Scheme of Preparation of a-, PSa-, and a-CM- fractions.



T-fraction throughout this paper, was Nakahara and Fukuoka's alcohol precipitate, and the second one called a-fraction was the fraction adsorbed on calcium phosphate gel from the water extract of tumor tissues, and the third called PSa-fraction was prepared in the same way as a-fraction by calcium phosphate gel absorption but from the boiled supernatant of tumor homogenate after removing a-fraction, and the last one which is called a-CM-fraction, the most active in catalase depressing action among these four preparations, was the fraction purified by the carboxy-methyl-cellulose column chromatography from a-fraction. The procedure of extracting each of the fractions is presented schematically in Table 1. and will be reported later in detail.

The activities of these preparations expressed as minimum dose per mouse to depress liver catalase activity *in vivo* were 75 mg, 20 mg, 10 mg, and 1 mg. for T-, a-, PSa- and a-CM-fraction respectively, the more quantitative assay data being presented in the section of Results in this paper.

Determination of Plasma Iron: To test the effect of toxohormone on plasma iron level, young female rats weighing around 100 g were used thoughout these experiments. 13 hours after the intraperitoneal injection of several graded doses of each test sample the blood was withdrawn with a syringe containing heparin from the abdominal aorta under pentbarbytulate anesthesia.

Plasma-bound iron and unsaturated iron-binding capacity of plasma were determined by the Nitroso-R reaction published by Noda *et al.* (7), which is accepted as the most sensitive color reaction of iron.

Assay of Liver Catalase Activity: The assay of liver catalase depressing activity of each toxohormone preparation was carried out using mice by the method employed routinely in this laboratory (2).

The Labeling of Plasma with ^{59}Fe: To the pooled plasma from several normal rats was added ^{59}Fe (0.2 μc per ml of plasma) in the form of $^{59}FeCl_3$ solution of high specific activity, and stood over 30 minutes at room temperature (1). All of the added ^{59}Fe was revealed to be bound tightly with β-globulin fraction of plasma protein by paper electrophoresis test.

Results

The Depression of Rat Plasma Iron by Each Toxohormone Preparation.

In Figure 1 are plotted the plasma iron levels of all the rats used in the assay of a-CM-fraction. The plasma iron level of the control female young rats kept on "Oriental" stock chow diet was found to be in a narrow range around 220 μg/dl. In this figure, it is clearly demonstrated that after 13 hours a-CM-fraction reduced the plasma iron level in a relatively small dose, and that 50% depression of plasma iron was achieved by the dose between 100–200 μg.

From the average of control group and each group injected with different doses of sample were calculated the 50% decrease of plasma iron at each dose, and the curves of percent decrease against log dose were constructed for T-, a-, PSa- and a-CM-fraction respectively, and shown in Figure 2. In all cases, each group for one dose of test sample consisted of not less than 3 rats. As generally seen in the percent inhibition-log dose curves, the curves with these four fractions revealed to be of sigmoid type, and the slopes of four curves were

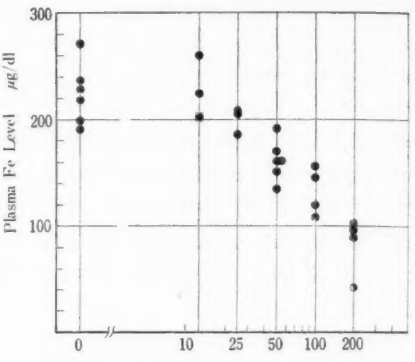


Fig. 1. Plasma Fe Level of Each Mouse 13 hrs. after the Injection of Various Doses of a-CM-fraction of Toxohormone, μg.

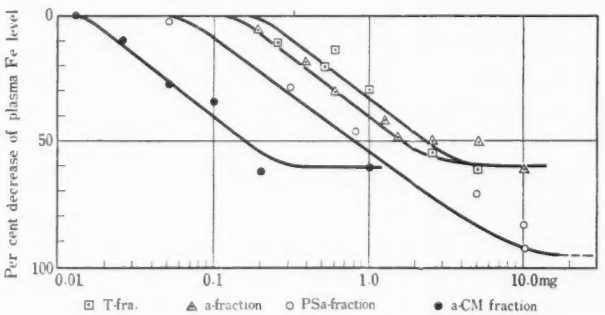


Fig. 2. Percent Decrease of Plasma Fe Level by the Injection of Various Doses of Each Sample.

quite parallel with each other. And these curves demonstrated that the maximum depression of plasma iron by T-, a-, and a-CM-fraction were 60%, while PSa-fraction in higher doses did depress the plasma iron almost completely. From these curves, 50% depressing doses were calculated as 2.2 mg, 1.6 mg, 0.78 mg and 0.15 mg per rats for T-, a-, PSa- and a-CM-fraction respectively.

Comparison of the Catalase Depressing Activities of Four Toxohormone Samples.

The order of the magnitude of activities of four samples to depress plasma iron level is just the same as that obtained semiquantitatively with liver catalase depression, but to establish a more quantitative relation, 50% catalase depressing doses of four preparations were determined as follows:

With T-fraction the relation between percent decrease of liver catalase activity and dose injected was studied most extensively, and practically the catalase depres-

sing activity of T-fraction was assayed at 5 dose levels ranging from 20 mg to 80 mg. In this range of doses the relation of percent decrease of catalase and log dose of T-fraction made a straight line as indicated in Figure 3. And the catalase depressing activities of the other three preparations of toxohormone, that is, a-, PSa-, and a-CM-fractions were assayed at two levels of doses. The line of percent decrease

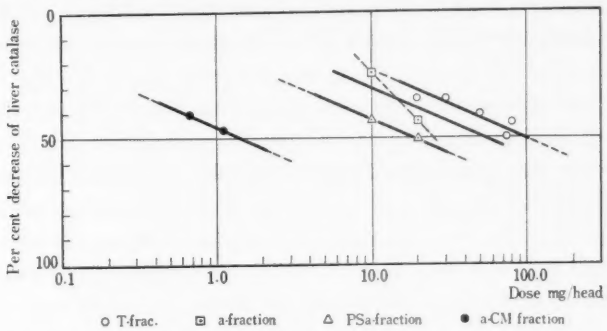
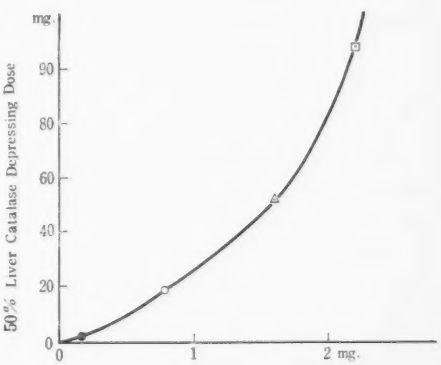


Fig. 3. Percent Decrease of Liver Catalase Activity by the Injection of Each Sample in Various Doses.

against dose in logarithm with each preparation is drawn also in Figure 3. In this figure, it may be noticed that all the lines, except one for a-fraction, were quite parallel to each other, especially to the well studied one of T-fraction. 50% catalase depressing dose of each sample was determined by intra- or extrapolation of each line, and the doses were 98 mg, 52 mg, 19 mg and 1.5 mg for T-, a-, PSa- and a-CM-fraction respectively.

The Relation between 50% Depressing Doses of Each Toxohormone Preparation for Liver Catalase and Plasma Iron Level.

The 50% catalase depressing dose was plotted against 50% plasma iron depressing dose with four samples tested in this experiment. As illustrated in Figure 4, the relation was not linear but was revealed [to be exponential.



	50% depressing dose	
	Plasma Fe	Liver Catalase
□ T-fraction	2.2	98
△ a-fraction	1.6	52
○ PSa-fraction	0.78	19
● a-CM-fraction	0.15	1.5

Fig. 4. Relation between 50% Depressing Doses of Toxohormone for Liver Catalase and Plasma Fe Level.

Thus the ratios of 50% catalase depressing dose to 50% plasma iron depressing dose were 45, 32, 24, and 10 for T-, a-, PSa- and a-CM-fraction respectively.

The Mechanism of Plasma Iron Depression by Toxohormone.

In the above experiments, it was confirmed that toxohormone preparations decrease the plasma iron level. As next step, experiments were carried out to examine whether the reduction of the rate to mobilize iron from its stock in the organs or the increase of the rate to remove plasma iron from blood is responsible for this phenomenon.

For this purpose, the plasma iron level was traced for as far as 13 hours after the injection of 10 mg of PSa-fraction, which was confirmed in the previous experiments to decrease the plasma iron almost completely. At 8 and 13 hours after the injection of PSa-fraction, 0.5 ml of ^{59}Fe labeled normal rat plasma containing 0.1 μc of ^{59}Fe was injected from femoral vein. At the same time control group without PSa-fraction was given the same dose of ^{59}Fe labeled plasma. One half of the rats of each group injected with labeled plasma was sacrificed 5 minutes after the injection, and the other half was sacrificed 1.5 hours after the labeled plasma administration. With each animal, the radioactivity of plasma, liver, spleen, femur bone and kidney were determined by a standard type γ-ray synchilation counter and the plasma iron level was measured as in the previous experiment.

The time course of changes in plasma iron level after the injection of PSa-fraction is presented in Figure 5. Plasma iron decreased gradually after the injection of PSa-fraction and at 13 hours it became nearly one-tenth of the control level.

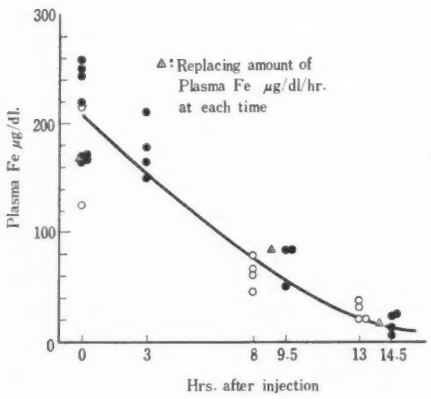


Fig. 5. The Time Course of Plasma Fe Level after the Injection of PSa-Fraction 10 mg Dose.

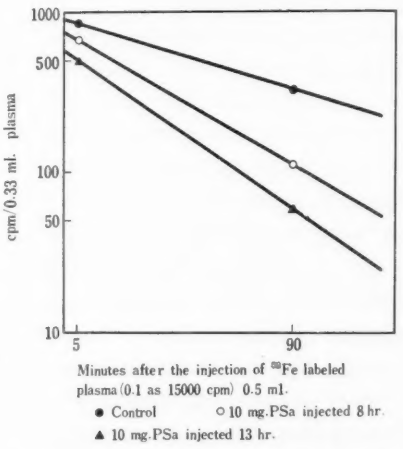


Fig. 6. Radioactivity of Plasma Fe after Injection of ^{59}Fe Labeled Plasma.

The average radioactivities of the plasma after 5 and 90 minutes in each group are presented in Figure 6. The three lines connecting the activities of 5 and 90 minutes points in Fig. 6 are for the control groups, 8 and 13 hrs. after PSa-fraction injection, from the top to bottom. Because the scale of radioactivity in Fig. 6 is logarismic the slope of each line represents the rate of clearance of labelled plasma from the blood. Comparing the slopes of these lines it can be seen that the removal rates of labelled plasma iron from blood is accelerated in toxohormone injected rats.

In Figure 7 are shown the radioactivities 1.5 hours after the injection of ^{59}Fe labeled plasma in each organ, that is, liver, spleen, femur bone and kidney of control, as compared with the group injected with PSa-fraction 8 hours and 13 hours beforefand. PSa injected group had a higher activity in liver and spleen and lower activity in kidney than the control, but in femur bone the counts were a little higher but the difference from control group was not significant.

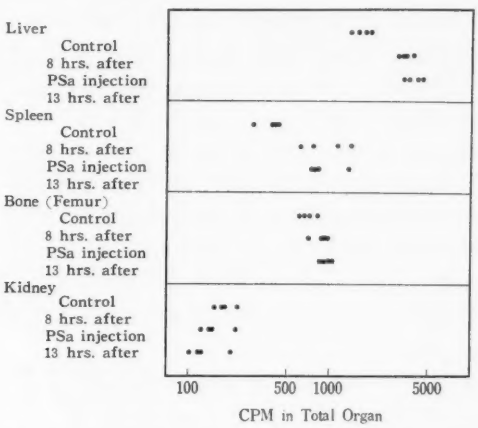


Fig. 7. Distribution of Radioactivity of ^{59}Fe in Rat Injected with PSa-Fraction.

Discussion

In these experiments, liver catalase depressing activities and plasma iron reducing activities of four different toxohormone preparations exhibited the same order of magnitude to each other, but the ratio between the two activities expressed by 50% depressing doses varied with each sample. However the relation between the two activities was not irregular but rather proportional as indicated in Fig. 4. Since the relation between the 50% depressing doses for liver catalase and plasma iron of those four samples showed a straight line on the graph of logarithmic scales, as illustrated in Fig. 8, the empirical formula for predicting one activity from the other

was constructed as

$$\log \phi = k \log \phi' + A \qquad (1),$$

where ϕ and ϕ' are the 50% depressing dose for mouse liver catalase and rat plasma iron respectively and k and A are constants to characterize the equation (1). Since we have found no theoretical ground for equation (1) as yet, we cannot apply it to new sample other than those we tested in this experiment, and at the present stage it is not possible to safely adopt the assay based on plasma iron level in place of the assay based on liver catalase.

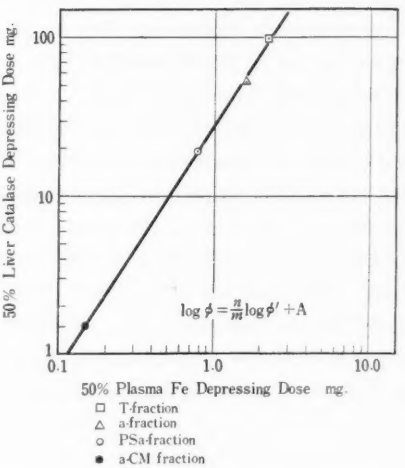


Fig. 8. Relation Between 50% Depressing Doses of Toxohormone for Liver Catalase ann Plasma Fe.

As to the mechanism of depressing plasma iron level by toxohormone the experiment using ^{59}Fe-labeled plasma revealed a acceleration of clearance from blood stream and retention in liver and spleen of the iron bound with plasma. In the interpretation of these results, however, one must consider the fact that after the injection of toxohormone preparation plasma iron level itself decreases rapidly and the increased rate of ^{59}Fe disappearance from blood does not reflect the increased amount of iron captured by tissues. The amount of plasma iron deprived from blood at each time after toxohormone injection could be calculated by using the data with ^{59}Fe as follows:

Let the specific activity of plasma iron be represented by y, and the decreasing rate by y, and $\frac{dy}{dt}$ can be assumed as

$$\frac{dy}{dt} = -\frac{b}{x} y \qquad (2), \text{ or}$$

$$y = C_1 e - \frac{b}{x} t \qquad (2'),$$

where x is plasma iron level, b plasma iron replacing amount per hour, C_1 the starting specific activity of plasma iron and t the time after the injection of ^{59}Fe-labeled plasma. In Equation 2′, we can discover the values of all the parameters included except b; thus the value of b, which is the replacing amount of plasma iron per hour, can be calculated for each group using equation 2′. In control rat group, $C_1 = 5.0$; y (after 1.5 hr.)$= 1.40$; $x = 200\ \mu$g/dl; $t = 1.5$ hr.; then b is calculated as 170 μg/dl/hr. With the group injected 3 hrs. before with PSa-fraction 10 mg, $C_1 = 11.2$; y (after 1.5 hr.)$= 1.47$; $x = 65\ \mu$g/d.; $t = 1.5$ hr; then b equals 88 μg/dl/hr. With the

group injected 13 hrs. before with PSa-fraction 10 mg, $C_1=20$; y (after 1.5 hr.)= 5.54; $x=20$ μg/dl.; $t=1.5$ hr.; then b becomes 17.1 μg/dl/hr. Thus, these calculations indicate that after the injection of PSa-fraction the amount of plasma iron deprived of by tissue from blood stream per hour decreased rapidly and remarkably as comparing to the initial level in control group, that is, at 8 hours it decreased to one-half and at 13 hours to one-tenth of the control group. Nevertheless, the decrease of plasma iron level means that the mobilization of the iron from tissue is reduced by the injection of PSa-fraction.

Although we cannot conclude absolutely that a single principle in the each toxohormone preparation is responsible for liver catalase depressing and plasma iron reducing activities, the mode of action of decreasing plasma iron is related to the reduced mobilization of iron from the tissues as revealed in these experiment, and this is very close to our postulation for the mechanism of depressing liver catalase activity. In this sense, we still hold to the idea that toxohormone has the actions of depressing both plasma iron level and liver catalase activity, at least until we find either a preparation which has only one activity or a means of dissociating these two actions of each toxohormone preparation.

Summary

All of the four toxohormone preparations tested in this experiment, which vary in the extracting procedures and in activities, were revealed to have the action of decreasing plasma iron level of rat. The order of magnitude of this activity of four preparations was just the same as that established for their liver catalase depressing activity. But the ratios of the effective doses for the two actions varied from sample to sample.

Concerning the mode of action of toxohormone in decreasing plasma iron it was demonstrated by using ^{59}Fe-labeled plasma that the lowering of iron mobilization from its tissue reserve may be the most probable mechanism.

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THE ISOLATION OF A BASIC PROTEIN HAVING TOXOHORMONE ACTIVITY FROM TUMOR TISSUES

SETSURO FUJII, TAKASHI KAWACHI, HIROMICHI OKUDA, BIN HAGA and YUICHI YAMAMURA
(Department of Biochemistry, Faculty of Medicine, Kyushu University)

INTRODUCTION

It is well known that the liver catalase activity of tumor-bering animals is significantly lower than that of normal animals (1-8). Nakahara and Fukuoka (9) (10) reported the isolation of a fraction from malignant tumor tissues which reduced the liver catalase activity within 24 hours when injected into mice and this liver catalase depressing factor was designated "toxohormone." This result suggested that certain toxic substance might be produced in tumor tissues which caused the decrease of the liver catalase activity of tumor-bearing animals. Greenfield and Meister (11) also confirmed the existence of the liver catalase depressing factor in tumor tissues and suggested that the active agent itself might possess or be associated with particles with a molecular weight of at least 40,000 from the dialysis and ultracentrifugal experiments. Ono et al. (12) subjected their toxohormone fraction to peptic digestion and isolated the crystalline picrate of basic polypeptide which had toxohormone activity in 0.2 mg dose for mice.

Recently, it was shown that toxohormone had not only the activities of depressing the liver catalase activity, but also of causing anemia, decreasing plasma iron level, increasing weight of liver, spleen, and adrenals, causing thymus atrophy (13) and suppress the biosynthesis of diphosphopyridine nucleotide of liver (14). Plasma iron was found to be more sensitive to toxohormone than liver catalase. In the present investigation, the basic proteins from human tumor tissues and rhodamine sarcoma were prepared and tested for their ability to lower liver catalase activity of mice and plasma iron level of rats after injection intraperitoneally into these animals.

MATERIAL AND METHODS

The following tumors were employed: 1) rhodamine sarcoma harvested 3 to 4 weeks after transplantation to rat, and 2) human hepatoma. All of them were carefully dissected free of necrotic areas.

Isolation of the basic protein from tumor tissue. Acetone powder was prepared by blending the tumor with 5 volumes of acetone in a Waring blender. The material

was filtered, washed with acetone and dried in vacuo. Acetone powder was suspended in ten volumes of 0.1 N HCl and allowed to stand for 2 days under stirring. The mixture was centrifuged and the precipitate was discarded. The pH of the supernatant was adjusted to 7 by the addition of 2 N NaOH and the insoluble material was removed by centrifugation to yield a clear solution. The supernatant was added

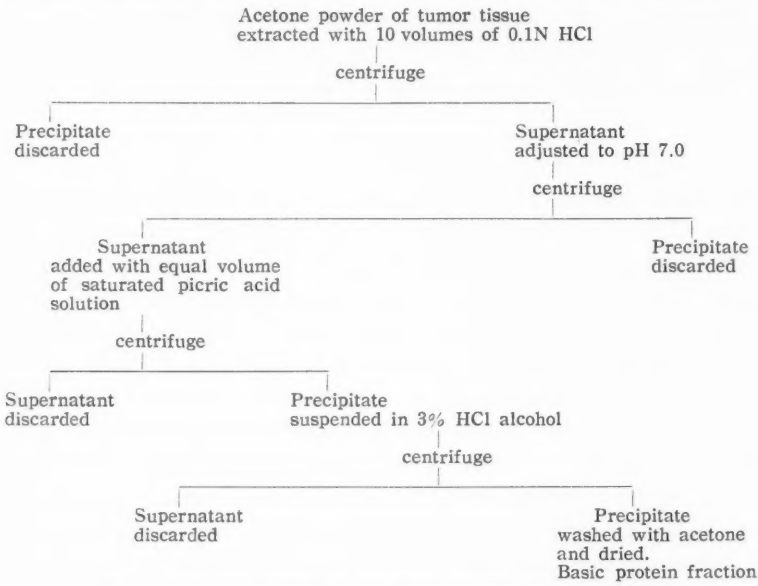

Fig. 1 Isolation method of the basic protein from tumor tissue.

with an equal volume of saturated picric acid solution and allowed to stand in refrigerator overnight. The resulted yellow precipitate was centrifuged, suspended in ten volumes of 3 per cent HCl alcohol and stirred overnight. The insoluble material was collected by centrifugation, washed with acetone and dried in vacuo. This fraction was designated "basic protein". An outline containing the essential features of this procedure is presented in Fig. I.

Assay. Catalase activity: the liver catalase activity of ddN (Kyudai) strain mice was determined at 24 hours following intraperitnoeal injection of the neutral solution of material to be tested. In this experiment, the mice older than 30 days after birth were employed except few cases in which insufficient number of animals were available, because the liver catalase activity of mice of this age is relatively constant (15). In each assay, the control animal of the same age injected with 0.85% sodium chloride was employed. The animals were sacrificed by decapitation and bled. The liver was quickly removed and the enzyme activity was determined by the method of Euler and Josephson (16).

Plasma iron: the rats of Wister-King strain were employed, their age being 50–100 days. At 12 hours after intraperitoneal injection of test material, the blood was obtained from the abdominal vein of narcotized rats and the amount of iron in plasma was determined by the method of Barkan and Walker (17).

Results

The basic proteins were isolated from rhodamine sarcoma and human hepatoma as described above. Their yields were almost the same in each case, being 4.1–4.5 % of acetone dried powder of tumor tissues.

The effect of injection of the basic protein of tumor tissue on liver catalase activity. Each material was tested in various doses and the results of these assays were given in Figs. 2 and 3.

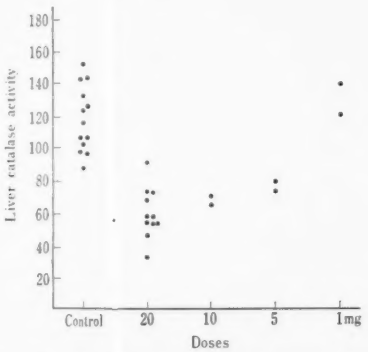

Fig. 2. Effect of various doses of the basic protein isolated from human hepatoma on the liver catalase activity of mice.

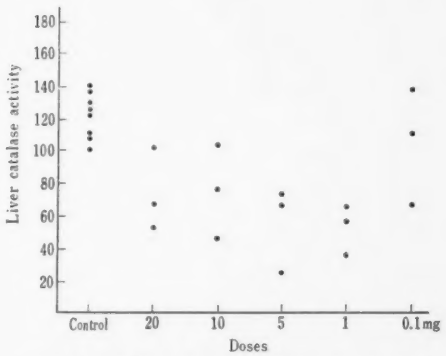

Fig. 3. Effect of various doses of the basic protein of rhodamine sarcoma on the liver catalase activity of mice.

The liver catalase activity of mice injected with basic protein isolated from human hepatoma and rhodamine sarcoma was less than that of control mice and the loss of catalase activity was of the same order of magnitude as that found in tumor bearing animals. The minimum effective doses of the basic proteins of rhodamine sarcoma and human hepatoma for catalase depressing activity were 1 mg and 5 mg respectively.

There are numerous chemical substances that act as inhibitors to catalase activity *in vitro*, while toxohormone isolated by Nakahara *et al.* has no activity *in vitro* experiment. The effect of basic protein on liver catalase activity *in vitro* was tested by the method of Nakahara (10). Liver catalase solution was prepared from the liver of normal mice by extracting 0.1 g of the liver homogenate with 10 ml of 0.1 M phosphate buffer (pH 7).

1.0 ml of liver catalase solution was added with 2.0 ml of buffer containing 25 mg of basic proteins, or the same amount of the phosphate buffer as control. The mixture was allowed to stand at room temperature for 60 minutes and the catalase activity was determined. As shown in Table 2, the basic proteins isolated from tumor tissues had no activity *in vitro*.

Table 1 Effect *in vitro* of the basic proteins on liver catalase activity.

Catalase solution ml	Buffer ml	Basic protein mg	Catalase activity
1.0	2.0	25.0	112.0
1.0	2.0	0	101.6

All conditions were as described in "results" section.

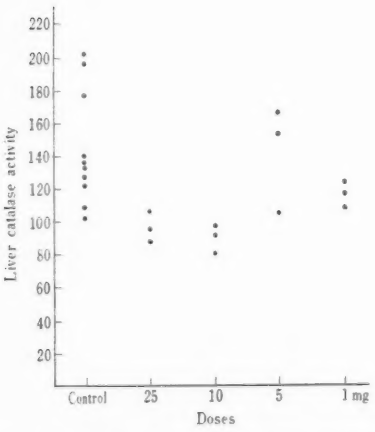

Fig. 4. Effect of various doses of the basic protein isolated from of normal rat liver on the liver catalase activity of mice.

dose of this material was required to be effective noticeably. The minimum effective dose of the basic protein of normal rat liver was 2–10 times that of tumor tissues.

The effect of injection of the basic proteins isolated from tumor tissues on plasma iron. The relationship between plasma iron level and body weight of normal rats was given in Fig 5. The data showed that the level of plasma iron did not

As the control experiment, the basic protein was isolated from normal rat liver by the same method described above and administered intraperitoneally into mice. As shown in Fig 4, the injection of the basic protein of normal rat liver also caused slight depression of liver catalase activity at 24 hours after injection and 10mg

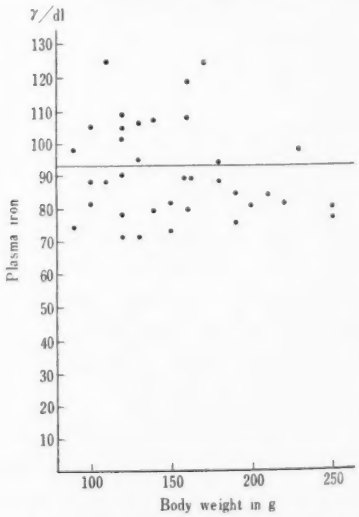

Fig. 5. The relationship between the level of plasma iron and body weight of normal rat.

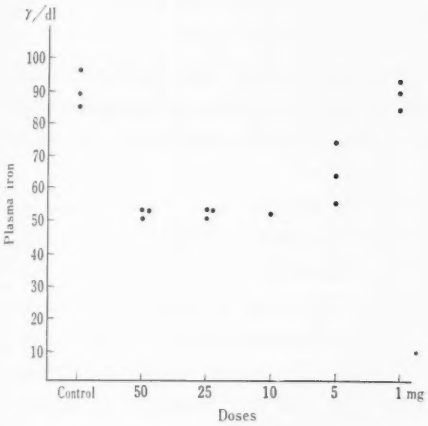

Fig. 6. Effect of various doses of the basic protein isolated human hepatoma on plasma iron of rat.

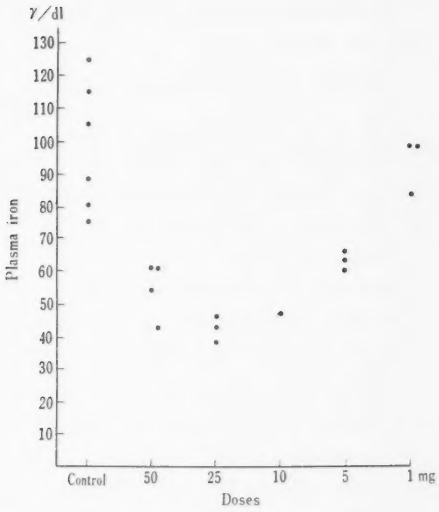

Fig. 7. Effect of various doses of the basic protein isolated from rhodamine sarcoma on plasma iron of rat.

alter in different body weight of rats and the averaged value was 93γ/dl. and there was scarcely any case of the level of plasma iron lower than 70γ/dl. In the course of these experiments, therefore, the test material was assumed to be active, when the level of plasma iron of animals was lower than 70γ/dl by the injection of material. The effect of injection of the basic proteins from rhodamine sarcoma and human hepatoma on plasma iron of rats was given in Figs. 6 and 7. Each material isolated from tumor tissues produced a marked decrease in the level of plasma iron at 12 hours after injection when only 5 mg of material was administrated, whereas 25 mg of the basic protein isolated from normal liver was required to reduce plasma iron level noticeably as shown in Fig. 8.

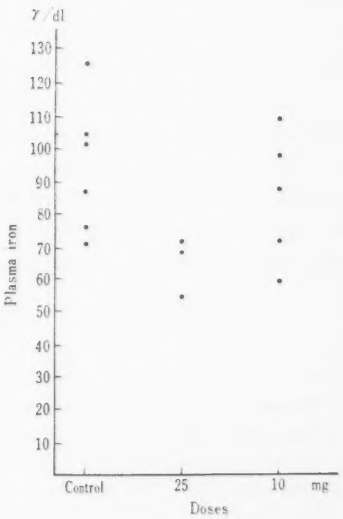

Fig. 8. Effect of various doses of the basic protein isolated from liver of normal rat on plasma iron of rat.

The minimum effective dose of the basic protein of normal rat liver was 5 times that of tumor tissues.

Discussion

The basic proteins which were isolated from human and experimental tumor tissues showed the toxohormone activities such as the depressing catalase activity of the liver of mice and the plasma iron level of rat by the intraperitoneal injection to these animals.

The material obtained from the normal tissue possessed little activity as compared with that derived from tumor tissues by the same procedure. These findings are compatible with the hypothesis established by Nakahara et al. (9) (10) that the lowered liver catalase of tumor-bearing animals is due to an inhibitory substance elaborated by the tumor and indicated that the active agent might be protein or some substance associated with protein. In *in vitro* experiment, the basic proteins of tumor tissues had no inhibiting action on catalase and this result is in agreement with toxohormone which was isolated by the method of Nakahara (10).

Summary

1. The basic proteins were isolated from human and experimental tumor tissues and their toxohormone activity was tested.

2. The liver catalase activity of mice was lowered within 24 hours following a single intraperitoneal injection of basic proteins.

3. The plasma iron level of rat was reduced within 12 hours following a intraperitoneal injection of basic proteins.

4. The basic protein obtained from normal tissues showed much less toxohormone activities than that of tumor tissues.

5. The catalase inhibiting activity of the basic proteins isolated from tumor tissues was not found in *in vitro* experiment.

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STUDIES ON LIVER CATALASE, β-GLUCURONIDASE, AND PLASMA IRON DURING DEVELOPMENT OF MICE

HIROMICHI OKUDA, BIN HAGA, TAKASHI KAWACHI, SETSURO FUJII and YUICHI YAMAMURA

(Department of Biochemistry, Faculty of Medicine, Kyushu University)

From the fact that glycolysis was remarkably active in embryo as well as cancer tissue, Warburg (1) proposed that cancer tissue was differentiated to embryonic type of tissue. Thereafter, many evidences supporting this hypothesis were found by embryologist and biochemist. In 1916, Blumenthal et al. (2) reported that catalase activity of cancer tissue was significantly lower than that of normal tissue and this finding was supported by Greenstein's work (3). On the other hand, Fishman (4), (5) demonstrated in 1947 that β-glucuronidase activity was very high in cancer and Heilmeyer (6) reported that plasma iron in tumor bearing animals showed low level.

In this communication, the change of the pattern of liver catalase, β-glucuronidase, and plasma iron level during development of ddN, CF1 and CFW strain of mice was studied.

EXPERIMENTAL

Animals: ddN (Kyudai), CF1 and CFW strain mice supplied by Japan Experimental Animals Research Association were used in this experiment.

Catalase activity: The method of Euler and Josephson (7) was employed to determine the catalase activity of liver and enzyme activity was expressed as velosity constant per 1 g of dry weight of liver.

Plasma iron: Blood was obtained from the abdominal veins of narcotized mice and the level of plasma iron was determined by the method of Barkan and Walker (8).

β-Glucuronidase: 200 mg of fresh liver tissue was removed from mice killed by decapitation, added to 2 ml of 0.85 per cent NaCl solution and ground in a glass homogenizer. The homogenate was diluted with 8 ml of 0.85 per cent NaCl solution. One tenth ml. of this solution was mixed with 0.1 ml of 0.1 M p-nitrophenol glucuronide and 0.8 ml of 0.2 N acetate buffer (pH 3.8). After the mixture was incubated for 3 hours at 38°C, 1 ml of 0.2 N NaOH was added into incubation mixture and the volume of solution was made up to 6 ml by the addition of water. Optical density was measured at 430 mμ by Beckmann spectro-photometer. The enzyme activity was expressed as mg of substrate hydrolyzed per 3 hours under the conditions of assay.

Results

The liver catalase activity of ddN (Kyudai) strain was measured at various times from embryonic life until 50 days after birth. As shown in Fig. 1, catalase activity rosed from low level before birth to maximum at around 30 days after birth and then maintained over the whole of the remaining period of the examination. The liver catalase activity of the adult mice was approximately 7 times that of embryo.

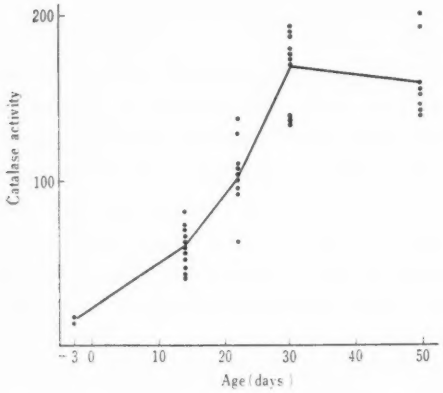

Fig. 1 Catalase activity of female mice liver at various ages.

The relationship between the body weight and the liver catalase activity of ddN female mice in the same age was presented in Fig. 2. The results indicated that the liver catalase activity of mice of the same age increased in proportion to body weight until the growth was established and no change of catalase activity of adult mice of the same age was observed in different body weight.

The liver β-glucuronidase activity of ddN strain female mice in relation to age was presented in Fig. 3 and the enzyme activity of infant mice was higher than that of adult and, as age increased, the activity declined to minimum at around 20 days and thereafter remained constant. The enzyme activity of infant mice was nearly 3 times that of adult.

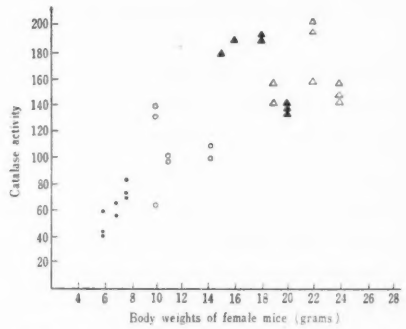

Fig. 2 The relationship between the liver catalase activity and the body weight of female mice.
● 14 days ○ 22 days ▲ 30 days
△ 50 days

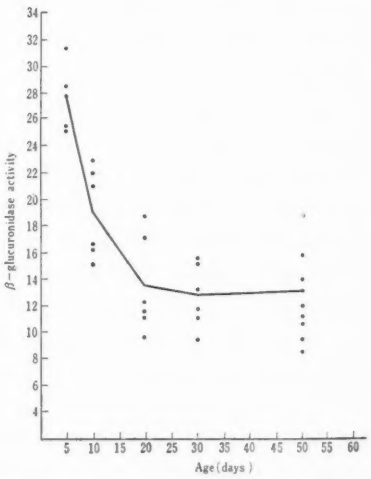

Fig. 3 β-Glucuronidase activity of liver of female mice at various ages.

Table 1 The liver catalase and β-glucuronidase activities of CF1 and CFW strain mice in various ages.

Enzyme	Catalase activity*						β-Glucuronidase activity*					
Strain	CFW			CF1			CFW			CF1		
Days after birth	28	35	45	28	35	45	28	35	45	28	35	45
♂	104	125	122	135	105	134	13.8	14.8	12.6	16.4	13.4	12.4
♀	90	118	122	119	118	118	13.8	13.2	12.8	12.5	13.4	11.3

* In each case, four mice were used and the values were averaged.

Table 2 The level of plasma iron of CFW and CF1 strain mice in various ages.

Strain		CFW			CF1		
Days after birth		28	35	45	28	35	45
Plasma iron* γ/dl	♂	112	132	112	112	105	129
	♀	100	143	99	117	128	103

* In each case, five mice were used and the values were averaged.

The liver catalase and β-glucuronidase activities of CF1 and CFW strain mice older than 28 days were measured and shown in Table 1. No noticeable change of these enzyme activities was observed in different age and sex during the period of examination.

The level of plasma iron was determined in CF1 and CFW mice older than 28 days. Five mice were used for each assay to obtain enough plasma to be tested. No change was observed also in difference of age and sex in these adult mice.

Discussion

The data obtained in the present work showed that the liver β-glucuronidase activity of infant ddN strain mice was higher than that of adult and this result was in agreement with the that of Levvy et al. (9). However, in similar experiment on rat, Mills et al. (10) reported that the infant rat liver β-glucuronidase activity was lower than that of adult. It must be concluded from these results that there is a species difference between mice and rats in the behaviour of liver β-glucuronidase activity as a function of age. The liver catalase activity of mice rosed, as age increased, to maximum at around 3 days after birth. As far as the present experiment is concerned, the pattern of enzyme activity of liver was strinkingly similar to that of cancer tissue.

Summary

1. Liver catalase activity of ddN female mice increased in proportion to age to maximum at around 30 days after birth and then remained constant.

2. Liver β-glucuronidase activity of ddN female mice declined from high level of infant mice to minimum at around 20 days after birth and thereafter maintained the constant value.

3. Catalase and β-glucuronidase activities of liver and plasma iron level in CFW and CF1 strain of adult mice were constant and there was no difference in different sex.

Acknowledgment

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ACYLASE ACTIVITY IN THE LIVER OF RATS FED 4-DIMETHYLAMINOAZOBENZENE

SANJI KISHI, KATSUHIKO HARUNO and BUN-ICHI ASANO
(Department of Biochemistry, Showa Medical School, Tokyo)

Four years ago one of the authors, Kishi, reported briefly some data on acylase, together with those on many other enzymes, in the review entitled "enzyme activity of rat liver during carcinogenesis." At that time the data about acylase were obtained manometrically by the Warburg technique, using acetanilide and diacetyl-L-tyrosine as substrates. Subsequently the authors continued the work by introducing Conway microdiffusion apparatus and made use of acetanilide and D, L-acetylmethionine as substrates. The enzyme sources employed were pathological liver, including hepatoma, the liver of fourth week of 4-dimethylaminoazobenzene (DAB) feeding and the liver of normal rat, i.e., the control liver. In this connection the regenerating liver and the liver of sham operated animal were also used for reference. Thus the present paper is an extension and supplement of the previous report on acylases.

Materials and Methods

Experimental animals. Albino rats of male sex of our laboratory stock were fed on the DAB diet, containing DAB at a level of 0.06 per cent, for about 150 days, then on normal diet for additional weeks. When these rats were autopsied, the livers showed already various grades of lesions in the liver. Then they were practically classified into two groups, namely, pathological but non-cancerous livers and hepatoma, and the former were subclassified into three, I, II and III, according to their increasing grades of cirrhosis.

Livers of rats fed on DAB in their fourth week of experiment were also employed in order to obtain the effect of DAB feeding, which might have acted upon liver enzyme.

Furthermore, the following animals were used, i.e., partial hepatectomy was performed on normal rats with the excision of about 40 per cent by weight of the liver, and the rats were sacrificed 48 hours following the surgery. The residual liver of the animal was used as the regenerating liver. The removed lobes at the time of operation was called the original liver and used as the control for the regenerating liver. The liver of rats, which had been merely laparotomized and sacrificed on the 48 th hour postoperatively, was used as that of sham operated rats.

Enzyme Preparation. The hepatic tissue was removed from the freshly sacrificed animal and was weighed and homogenized with Krebs-Ringer solution at pH 7.4 or simply with distilled water in adequate dilution of fresh hepatic tissue. Then the homogenate was filtered through cotton wadding and used as an enzyme source.

An aliquot of the homogenate was dried and weighed for the calculation of enzyme activity against dried weight, taking into consideration the medium, when Krebs-Ringer solution was used.

Assay of acylase activity by Warburg manometric technique. The CO_2 evolution accompanied with the enzyme reaction was measured by a Warburg flask in a water thermostat maintained at 37.5°C, under the gas phase of a mixture consisting of 95 per cent N_2 and 5 per cent CO_2 volumetrically.

In the main compartment of the reaction flask were placed 1 ml of enzyme preparation, 5 times diluted with Krebs-Ringer solution, and 2 ml of Krebs-Ringer solution. To the side arm of the flask was brought in 1 ml of substrate solution, i.e., an aquous solution of 0.05 M acetanilide saturated with CO_2, and was tipped into the main compartment after 15 minutes equilibration.

Control flasks were assembled with all components other than the substrate solution or enzyme preparation, these having been replaced by an equivalent amount of CO_2-saturated distilled water or Krebs-Ringer solution, respectively. In all determinations a thermobarometer was employed for correction for alternations in temperature and atmospheric pressure.

The acticity was calculated from the initial linear portion of the curve and expressed as CO_2 evolution in cmm per hour per mg of dried tissue homogenate (Qco_2).

The measurement was carried out at every 10 minutes for 1 hour after the process has been started and the value at 30 minutes was multiplied by 2 to obtain 1-hour value.

In the case of acylase assay, by using diacetyl-L-tyrosine as substrate, which has been prepared from L-tyrosine and ketene after the method of Bergmann and Stern. The method of determination was nearly the same as described in the above lines except the following points.

The enzyme source used for this study was a homogenate diluted 100 times with Krebs-Ringer solution, and the 0.05 M aquous solution of the substrate was firstly adjusted approximately to neutral by NaOH and then was saturated with CO_2.

Assay of acylase activity applying Conway unit. In the beginning the digestion was carried out in small test-tube, into which were placed 2 ml of 0.1 M phosphate buffer solution at pH 7.2, 1 ml of tissue homogenate, diluted 10 times with water, and 1 ml of 0.1 M acetylmethionine, which has been adjusted to neutral. The reaction mixture was stoppered and incubated for 2 houres at 37°C. At the end of the time the test-tube was immediately dipped into ice-water and 1 ml of 0.4 M citric acid

was added, so the volume of the digestion mixture became 5 ml in total. One ml of the above mixture was transferred uniformly into the outer ring of Conway unit, where on the floor 7–8 g anhydrous sodium sulfate has been laid. Into the central well of the unit has previously been pippeted 2 ml of distilled water, the greased glass plate was put on it, and placed overnight at room temperature. After openning the unit, the titration of the aqueous portion of the unit was carried out immediately with 1/300 N $Ba(OH)_2$ solution by using phenol red as indicator under the current of CO_2-free air. The activity was represented by the ratio of 5 times the titration amount (mcc) to dried weight (mg) of 1 ml tissue homogenate.

In the case of acetanilide test, the reaction mixture in a test-tube consisted of 2 ml of 0.1 M phosphate buffer solution, 2 ml of 0.05 M acetanilide solution, which was adjusted to neutral prior to use, and 1 ml of homogenate in 10 times dilution. The further procedure was similar to the case in which acetylmethionine was tested. In this case after the addition of 1 ml of 0.4 M citric acid into the digestion mixture, the total volume of which became 6 ml.

The activity of acetanilide acylase was represented by the ratio of 6 times the titration amount (mcc) to the dried weight (mg) of 1 ml tissue homogenate.

Results and Discussion

Acylase activity, using acetanilide as substrate, in the liver of rats fed DAB was measured by the Warburg technique and the distillation method applying Conway units, and the results obtained from the two methods coincided with each other (Table 1, Fig. 1) (Table 3, Fig. 3).

Normal liver and pathological but non-cancerous liver, even with severe cirrhosis possessed a fair amount of digestive activity, whereas once the liver turned into cancerous the activity diminished to zero, so far as can be determined by the above methods.

The liver at the fourth week of DAB feeding showed appreciablly higher activity than normal liver. In the case of regenerating liver the activity diminished to about a half value of the excised portion of the same liver, obtained at the time of partial hepatectomy, and there were no change of activity in the liver of sham operated animal (Fig. 3').

When diacetyl-L-tyrosine was tested by applying Warburg manometer, pathological livers including hepatoma showed higher activity than normal liver, especially in the case of marked cirrhotic liver. The liver of DAB rats in their fourth week of experiment showed also higher activity (Table 2, Fig. 2).

When acetylmethionine was studied by Conway units, acylase of the pathological but non-cancerous livers showed higher activity than normal liver. In the case of hepatoma, however, the activity was diminished to 60 per cent of that of normal

liver (Table 4, Fig. 4).

Table 1. Acylase activity in the liver of rats using acetanilide as substrate and measured by Warburg technique

Normal rats	DAB rats				
	Liver findings in long period of feeding				Liver in fourth week of feeding
	Pathological but non-cancerous liver			Hepatoma	
1.61±0.45 (10)	I	II	III		
	1.83±0.31 (5)	1.79±0.85 (6)	1.49±0.54 (6)	0 (3)	1.88±0.30 (6)

Table 2. Acylase activity in the liver of rats using diacetyl-L-tyrosine as substrate and measured by Warburg technique.

Normal rats	DAB rats				
	Liver findings in long period of feeding				Liver in fourth week of feeding
	Pathological but non-cancerous liver			Hepatoma	
63.0±9.6 (10)	I	II	III		
	75.1±7.5 (4)	108.7±29.1 (4)	96.4±29.8 (4)	76.2±15.4 (5)	90.9±27.3 (3)

Table 3. Acylase activity in the liver of rats using acetanilide as substrate and measured by Conway method.

Normal rats	DAB rats				
	Liver findings in long period of feeding				Liver in fourth week of feeding
	Pathological but non-cancerous liver			Hepatoma	
56±9.4 (7)	I	II	III		
	62 (1)	84 (1)	67±7.4 (5)	1±1.4 (5)	71±7.2 (4)

Table 4. Acylase activity in the liver of rats using acetylmethionine as substrate and measured by Conway method.

Normal rats	DAB rats				
	Liver findings in long period of feeding				Liver in fourth week of feeding
	Pathological but non-cancerous liver			Hepatoma	
130±25.3 (9)	I	II	III		
	170 (1)	135±28.5 (3)	148±19.9 (5)	79±22.5 (4)	145±35.0 (3)

Explanation for Tables 1 and 2: Cipfers are represented by CO_2 evolution in mcc/hr/mg dried tissue homogenate. Mean values with standard deviations and number of animals in parentheses for corresponding animals. Roman numerals indicate the grade of lesions of pathological but non-cancerous liver.

Explanation for Tables 3 and 4: Cipfers are represented by the ratio of 6 times (Table 3) or 5 times (Table 4) the titration amount of 1/300 N $Ba(OH)_2$ solution in mcc to dried weight in mg of 1 ml tissue homogenate. Mean values with standard deviations and number of animals in parentheses for corresponding animals. Roman numerals indicate the grade of lesions of pathological but non-cancerous liver.

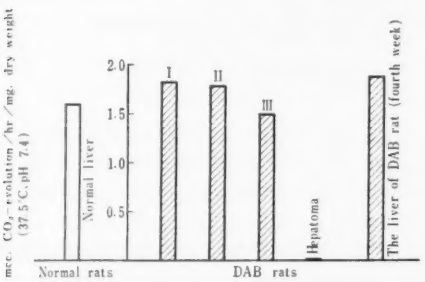

Fig. 1 Acylase activity in the livers of DAB rats
Substrate: acetanilide
Measuring method: Warburg technique

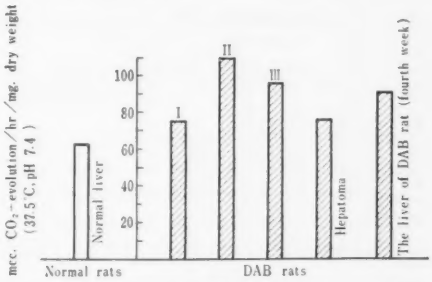

Fig. 2 Acylase activity in the livers of DAB rats
Substrate: diacetyltyrosine
Measuring method: Warburg technique

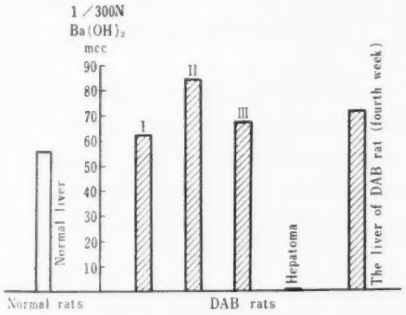

Fig. 3 Acylase activity in the livers of DAB rats
Substrate: acetanilide
Measuring method: Conway microdiffusion method

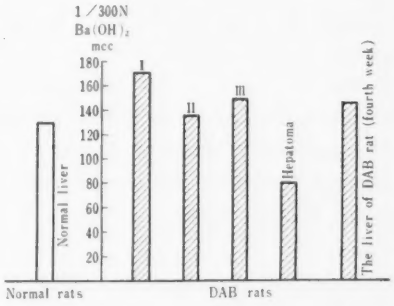

Fig. 4 Acylase activity in the livers of DAB rats
Substrate: acetylmethionine
Measuring method: Conway microdiffusion method

Figs. 1–4. The data in Tables 1–4 are represented graphically as heights of bars in Figs. 1–4, respectively. Unshaded bars represent the acylase activity of normal rat-liver and shaded bars represent that of the livers of DAB rats. Roman numerals indicate the grade of liver lesions as in the tables.

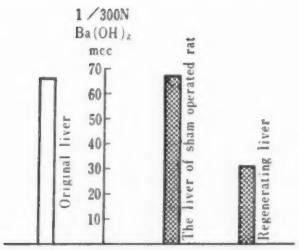

Fig. 3′ Acylase activity in the regenerating liver
Substrate; acetanilide
Measuring method: Conway microdiffusion method

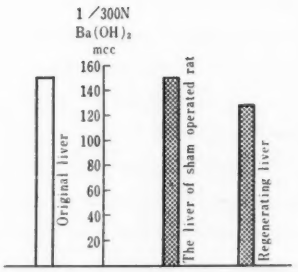

Fig. 4′ Acylase activity in the regenerating liver
Substrate: acetylmethionine
Measuring method: Conway microdiffusion method

Figs. 3′–4′. The height of bars are represented by the data, which are obtained similarly as describe in the explanation for Tables 3–4. The regenerating liver (right stippled bar) is compared with the liver of sham operated rat (left stippled bar) and original liver (unstippled bar).

The regenerating liver and the liver of sham operated animal showed also similar value to that of normal liver including the excised liver at the time of partial hepatectomy (Fig. 4′).

The effect of fed DAB on the acylase activity was detected in the liver of rats in their fourth week of experiment and there was seen no evidence of inhibition in every test case of different substrates, and the effect seemed to be accelerating especially in the case of diacetyl-L-tyrosine test.

It was noteworthy that the activity of acylase in hepatoma showed variable behavior according to the substrates used. When acetanilide was tested, the activity was impossible to measure, and in the case of diacetyl-L-tyrosine test, the activity increased more than the normal value, and in the case of acetylmethionine test the activity showed roughly a half value of that of the normal liver as described already.

The authors postulate the following explanation for the observed phenomena; there are some acylases in the normal liver and each keeps its own activity probably in a balanced state, but when the liver changes from normal to cancerous, the activity of each acylase might turn into an unbalanced state as a part of the metabolic systems in hepatoma.

Sammary

1) Activity of acylase in the liver of rats fed 4-dimethylaminoazobenzene (DAB) was measured by using as substrates acetanilide (AA), diacetyl-L-tyrosine (DAT)

and acetylmethionine (AM).

2) Activity of AA acylase in the slightly cirrhotic liver was higher than that of normal liver and even the severe case showed neary the same height as the normal value, whereas the activity in hepatoma was scarcely detected.

3) When DAT was used for acylase test, pathologically changed livers, including hepatoma, showed higher activity than normal liver.

4) AM acylase showed slightly higher activity in the pathological but non-cancerous livers than normal value. Hepatoma showed nearly the half value of that.

5) The liver of DAB rats in their fourth week of experiment showed higher activity than normal, tested with any of AA, DAT and AM.

6) Regenerating liver possessed somewhat lesser activity in the test of AM and half value in that of AA.

This investigation was supported in part by a grant-in-aid from the Japanese Ministry of Education.

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[GANN, 51, 243~248; September, 1960]

PAPER ELECTROPHORETIC STUDIES ON ENZYMES IN THE LIVER OF RATS FED 4-DIMETHYLAMINOAZOBENZENE V. ACYLASE

SANJI KISHI, BUN-ICH ASANO and YUTAKA TAMURA
(Department of Biochemistry, Showa Medical School, Tokyo)

As one of the series of works going on in our laboratory at present, a further study on acylase in the liver of rats fed 4-dimethylaminoazobenzene (DAB) was carried out in detail by introducing the method of paper electrophoresis.

Materials and Methods

Experimental animals. DAB rats. The male rats about 100 g in weight of our laboratory stock were maintained on the DAB diets at a concentration of 0.06 per cent for up to 150 days, after which the rats were removed from the DAB diet and replaced on normal diet for further weeks. When autopsied the liver of rats showed various grades of pathological changes including hepatoma. DAB rats in their fourth week of experimental days were also employed.

Normal rats used were of similar weight which have been maintained solely on normal diet.

Materials. The hepatic tissues were removed from freshly sacrificed animals and were then homogenized with three times the weight of distilled water and then subjected to paper electrophoresis.

Methods. Onto the paper 0.01 ml of homogenate was applied for each 1 cm of the initial line, under the conditions of 400 volts and the current of 2.5 ma by using 0.1 M veronal acetate buffer at pH 8.4. After 15 hours of electrophoresis the paper strip was removed and was cut into narrow oblongs with 1 and 2 cm sides.

The acylase activity was measured by using acetanilide as substrate. Into one series of some 20 small test-tubes were transferred 2 ml of a substrate-buffer solution each, which was prepared by mixing 20 ml of 0.5 M acetanilide solution and 30 ml of 0.25 M phosphate buffer at pH 7.2.

A paper segment of the size of 1×2 cm was delivered into each test-tube, which was stoppered and incubated at 38°C for 3 hours. At the end of this time the test-tubes were cooled instantly in ice-water and 0.5 ml of 0.4 M citric acid solution was then added to each test-tube.

One ml of the content of each test-tube was brought into the outer ring of the Conway unit, in which 7-8 g of anhydrous sodium sulfate has been laid. In the

central well of the unit has previously been kept 2 ml of distilled water. Then the greased lid was fixed in proper position tightly and the units were kept overnight at room-temperature.

After openning the unit the titration of aquous portion was carried out with 1/300 N $Ba(OH)_2$, using phenol red as indicator under the CO_2 free air-current.

The enzyme activity was reprensented by the number of mcc of the baryta solution used for titration, and calculated per paper segment of the size of 1×1 cm, and per 1 hour of incubation for digestion.

The activity was represented graphically to facilitate further discussion. On the ordinate the activity was shown by the above explained amount and the abscissa was divided in cm to plot the actual length of electromigration on the paper. When these points were plotted, an activity pattern was obtained.

In the measurement of acylase activity using D, L-acetylmethionine as substrate, the preparation of substrate-buffer solution was prepared by mixing 10 ml of neutralized 0.1 M D, L-acetylmethionine and 40 ml of 0.025 M phosphate buffer at pH 7.2, and the duration of digestion was 2 hours at 38°C. Further procedures and the measurement of the enzyme activity and its representation of its pattern were quite the same as described in the foregoing lines.

Results and Discussion

The patterns of acylase activity in the livers of rats fed the carcinogen, using

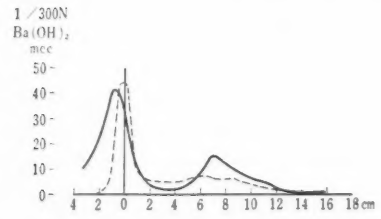

Fig. 1 Normal liver.

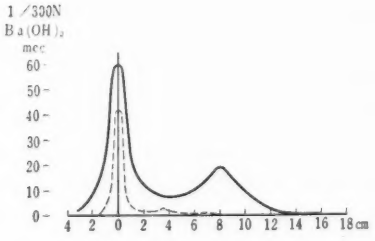

Fig. 2 Normal liver.

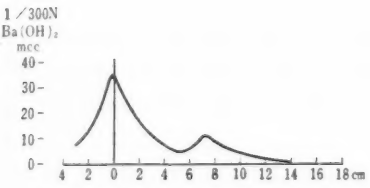

Fig. 3 Normal liver.

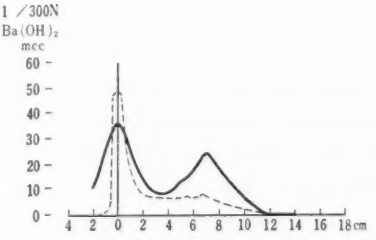

Fig. 4 Cirrhotic liver.

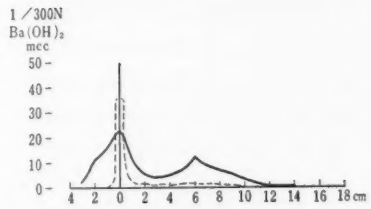

Fig. 5 Cirrhotic liver

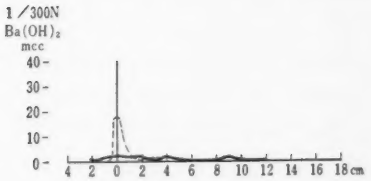

Fig. 6 Hepatoma.

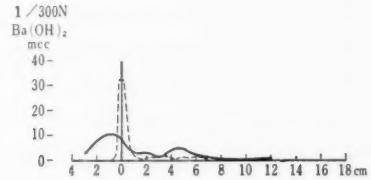

Fig. 7 Hepatoma.

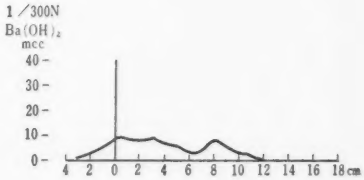

Fig. 8 Hepatoma.

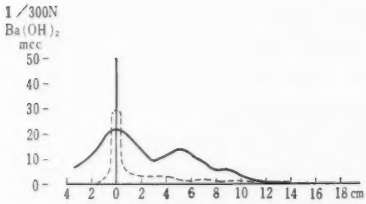

Fig. 9 The liver of DAB rat (fourth week)

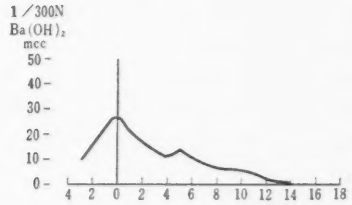

Fig. 10 The liver of DAB rat (fourth week)

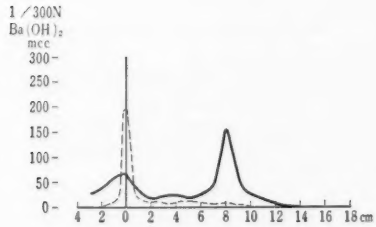

Fig. 11 Normal liver.

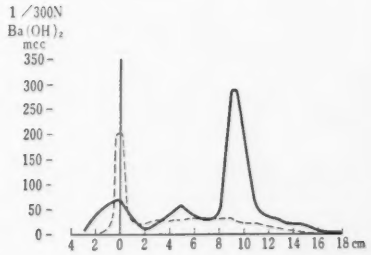

Fig. 12 Normal liver.

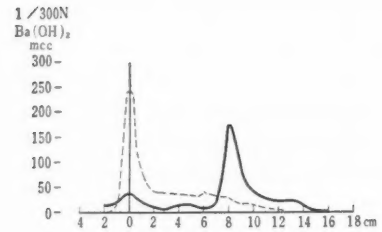

Fig. 13 Cirrhotic liver.

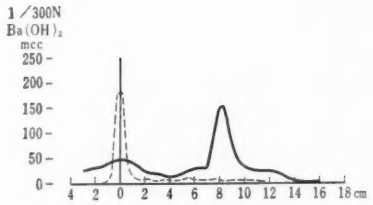

Fig. 14 Cirrhotic liver.

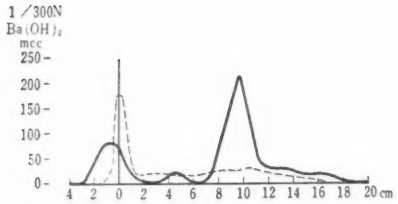

Fig. 15 Hepatoma.

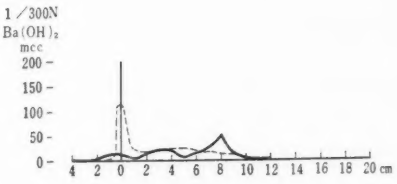

Fig. 16 Hepatoma.

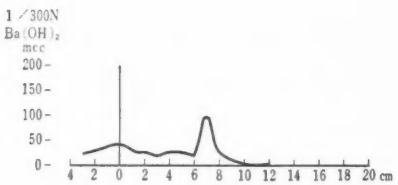

Fig. 17 Hepatoma.

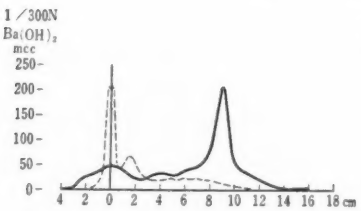

Fig. 18 The liver of DAB rat (fourth week)

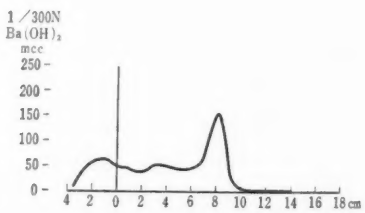

Fig. 19 The liver of DAB rat (fourth week)

Figs. 1-19. Activity patterns of acylase in the hepatic tissues of DAB rats are demonstrated with those of the normal rat-liver in heavy curves in Figs. 1-19.

Acetanilide was used as substrate in the cases of Figs. 1-10 and D, L-acetylmethionine in those of Figs. 11-19.

Broken curves indicate the distribution patterns of protein in arbitrary units, which are measured by the stained strips of corresponding paper electrophoresis.

acetanilide and D, L-acetylmethionine as substrates, were demonstrated with the aid of paper electrophoresis.

Firstly the acylase activity of acetanilide came into question. The pattern showed two well separated peaks in the case of normal liver, i.e., one sharp and high peak was shown at the starting line and the other lower one at the electromigrated part of protein (Figs. 1-3). The prominent peak at the starting line in the pattern of cirrhotic liver became flat, so the level-difference between two peaks was reduced (Figs. 4-5).

The patterns obtained from hepatoma showed curves of low level. The characteristic two peaks, which were seen in the cases of normal and cirrhotic liver, were lost nearly completely (Figs. 6-8).

The patterns showed some different appearance in the case of the liver of rats in their early experimental days (fourth week) (Figs. 9-10), although there were two lowered peaks at their proper positions; they were somewhat confluent so to speak. So the enzyme in the liver might has been affected by continuously fed DAB, that it became recognizable in the activity pattern.

There were three peaks in the activity patterns in the case of normal liver, when acetylmethionine was tested (Figs. 11-12). Two low peaks were situated on the starting line and at moderate moving part of protein zone respectively. The third one, a sharp prominent peak, was located in the rapidly moving component.

The patterns of cirrhotic liver (Figs. 13-14) and hepatoma (Figs. 15-17) showed the same character as in the case of the normal liver, although the patterns of hepatoma had a tendency of lowering of the peak at the rapidly moving component.

The patterns of the liver of DAB rats in their early stage of experiment (fourth week) (Figs. 18-19) possessed peaks with rather confluent appearance just as in the case of the analogous pattern, which was obtained by using acetanilide (Figs. 9-10).

According to the essentially different feature of patterns by the different substrates, especially obvious in the tests of normal liver, the authors might suggest that there exist many sorts of acylases in the category of the enzyme generally called acylase.

Summary

1) Activity patterns of acylase in the homogenate of the liver of rats fed 4-dimethylaminoazobenzene were demonstrated with the aid of paper electrophoresis using acetanilide and D, L-acetylmethionine as substrates.

2) The patterns showed a different feature according to the used substrate, i.e., 2 peaks were recognized in the pattern, when acetanilide was tested and 3 were found in the case of acetylmethionine test.

3) No essential difference was found between the patterns of the normal and cirrhotic liver.

4) In the patterns of hepatoma the characteristic peaks were still maintained to some extent, but they disappeared sometimes by flattening in the case of using acetanilide.

5) The patterns obtained from the liver of the carcinogen fed rats in their fourth week of experiment showed the ambiguous separation of the peaks.

This investigation was supported in part by a grant-in-aid from the Japanese Ministry of Education.

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[GANN, 51, 249~253; September, 1960]

PAPER ELECTROPHORETIC STUDIES ON ENZYMES IN THE LIVER OF RATS FED 4-DIMETHYLAMINOAZOBENZENE VI. ACETYLCHOLINESTERASE

BUN-ICHI ASANO
(Department of Biochemistry, Showa Medical School, Tokyo)
(Director: Prof. Sanji Kishi)

Studies on acetylcholinesterase (AChE) was added as one of the series to our work on various enzymes in the liver of rats fed 4-dimethylaminoazobenzene (DAB), by using the method of paper electrophoresis. The mere measurement of this enzyme activity was previously worked out by Sato in the laboratory of the present author and rise and fall of the activity during hepatocarcinogenesis was discussed.

Materials and Methods

Experimental animals. DAB rats. The male rats, weighing about 100 g, of our laboratory stock were kept on the DAB diet at a concentration of 0.06 per cent for 150 days, after which were removed from DAB diet and replaced on normal diet for additional weeks. When autopsied the liver of these rats showed various grades of pathological changes including hepatoma. The normal-appearing livers which were obtained from the rats in their early stages of DAB feeding were also used. Untreated rats which have been kept on basal diet were used as normal rats.

Materials. The hepatic tissues were removed from freshly sacrificed animals and were then homogenized with three times the weight distilled water and then subjected to paper electrophoresis.

Onto each 1 cm of the initial line of the paper 0.01 ml of homogenate was applied, under the conditions of 400 volts and the current of 2.5 ma, using 0.1 M veronal acetate buffer at pH 8.4. After 15 hours of electrophoresis the paper strip was removed and was cut into squares with 1 cm sides and was used as enzyme sources.

Blood serum. Blood was obtained by cardiac puncture from normal rat as well as presumably hepatoma-bearing DAB rat, under light anesthesia prior to sacrifice.

Sera were separated from blood corpscles, 0.01 ml of which was introduced without dilution to the apparatus of paper electrophoresis for each 1 cm of the initial line, and the conditions of electrophoresis were entirely the same as described above.

Measurement of AChE activity. The digestion was performed in series of about 20 small test-tubes, each containing 2 ml of the substrate-buffer mixture, which was prepared by dissolving acetylcholine chloride in 0.25 M phosphate buffer at pH 7.2 to 0.005 M solution.

Into each test-tube a paper segment of the size of 1×1 cm was put as the enzyme source, stoppered and incubated at 38°C for 2 hours. At the end of the time the test-tubes were dipped into ice-water without delay and to each test-tube was added 0.5 ml of 0.4 M citric acid solution to interrupt the digestion instantly.

One ml of the content of each test-tube was transferred uniformly into the outer ring of Conway unit, where 7-8 g anhydrous sodium sulfate has been spread. In the central well of the unit was put 2 ml of distilled water beforehand and greased lid was put tightly in proper position and placed overnight at room-temperature. Immediately after openning the unit, the aquous portion of the unit was titrated with 1/300 N $Ba(OH)_2$ solution by using phenol red as indicator. CO_2 free air was sent over the unit while carrying out the titration.

The activity of the enzyme was represented by the number of mcc of the baryta solution used for the neutralization of acetic acid, which has been liberated from 1 ml of substrate-buffer mixture for 1 hour of incubation at 38°C by the enzyme soaked paper segment of 1×1 cm size.

The activity was illustrated graphically as a pattern by plotting the activity on the ordinate and the actual displacement in cm on the abscissa.

Results and Discussion

The activity patterns of AChE obtained from the normal liver (Figs. 1-2) showed three peaks in all, fairly well separated, i.e., one prominent peak on the starting line and other two peaks at the moderate and fast moving parts, respectively.

In the case of cirrhotic liver (Figs. 3-4) and even of hepatoma (Figs. 5-6), patterns showed also three peaks of similar type as in normal liver. A magnified peaks were found sometimes in the pattern of hepatoma caused by its high activity of AChE (Fig. 5).

The liver of rats in their early experimental days (fourth week) (Figs. 7-8) showed somewhat ambiguous pattern. This pattern possessed one point in common with that of other hepatic tissues, i.e., the peak on the starting line. AChE might has

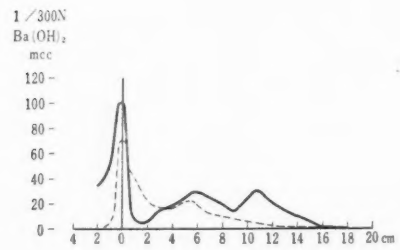

Fig. 1 Normal liver.

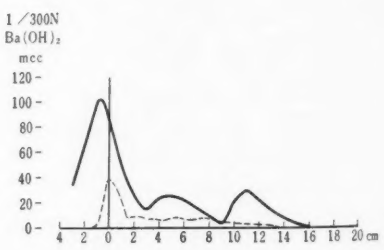

Fig. 2 Normal liver.

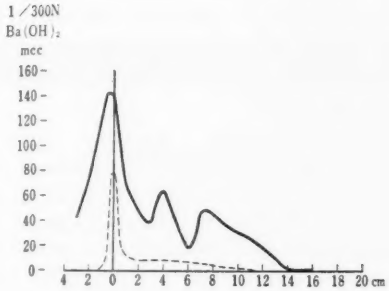

Fig. 3 Cirrhotic liver.

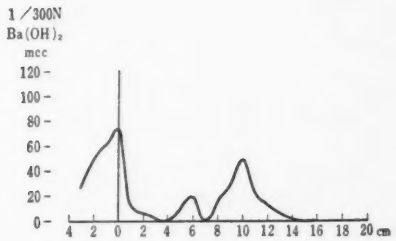

Fig. 4 Cirrhotic liver.

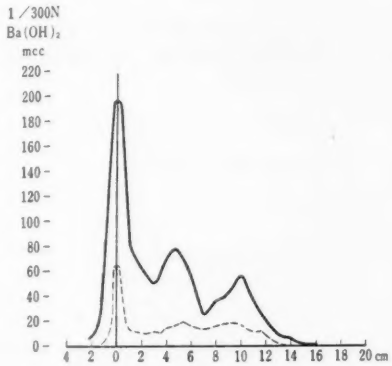

Fig. 5 Hepatoma.

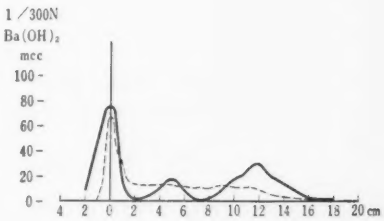

Fig. 6 Hepatoma.

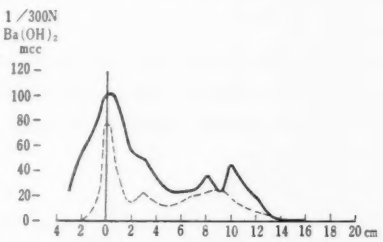

Fig. 7 The liver of DAB rat (fourth week)

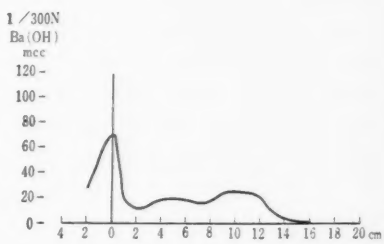

Fig. 8 The liver of DAB rat (fourth week)

Figs. 1–8. Activity patterns of acetylcholinesterase in the hepatic tissues of DAB rats are shown with those of normal rat-liver in solid curves.

Broken curves indicate the distribution patterns of protein in arbitrary units, which are measured by the stained strips of corresponding paper electrophreis.

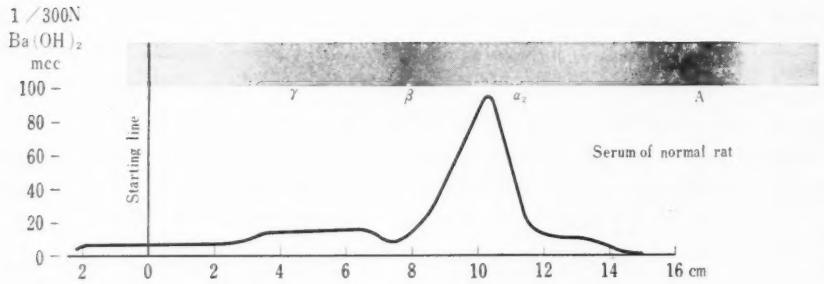

Fig. 9 Serum of normal rat

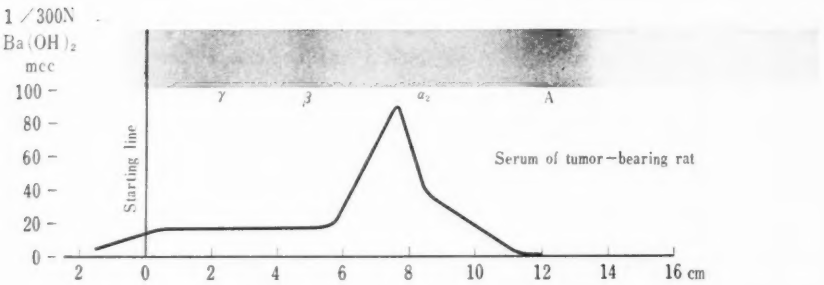

Fig. 10 Serum of tumor-bearing rat.

Figs. 9 and 10. Activity patterns of acetylcholinesterase in blood sera of normal rat and hepatoma-bearing rat, with the stained strips of corresponding paper electrophoresis.

been affected in the liver of rats by continuous DAB feeding, and it may be visualized as a deformation of activity patterns.

The pattern of AChE activity in normal rat serum (Fig. 9) showed one prominent peak between α_2- and β-globulin fractions and a low plateau extended from the starting line to the foot of the peak. No remarkable change could be detected in serum of tumor-bearing rat (Fig. 10).

Summary

1) Activity patterns of acetylcholinesterase in the liver of rats fed 4-dimethylaminoazobenzene were demonstrated with the aid of paper-electrophoresis.

2) Three peakes were observed in the patterns and there was found no essential difference among the patterns of the normal liver, cirrhotic liver and hepatoma.

3) The activity pattern of the liver of rats on their fourth week of the carcinogen feeding showed uncertain separation of peaks.

4) The activity pattern in sera of rats showed one sharp peak between α_2- and

β-globulin fractions and no differences was found between the sera of normal and hepatoma-bearing rats.

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[GANN, 51, 255~264; September, 1960]

BEHAVIOR OF THE PROTEIN-BOUND DYE, N-DEMETHYLASE, CATALASE AND XANTHINE OXIDASE ACTIVITIES OF RAT LIVER DURING THE COURSE OF REPEATED ADMINISTRATIONS OF 3'-Me-DAB

MITSUO MATSUMOTO, TOSHIO YAMADA and HIROSHI TERAYAMA
(Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo)

INTRODUCTION

Many biochemical changes observed in animal tissues during the experimental induction of tumors have been reported. Generally speaking, the observed biochemical changes are considered to fall into one of the following three types: 1) Gradual increase[1], 2) gradual decrease[2,3] and 3) showing a maximum or minimum [4,5,6] in the course of the administration of carcinogens. The biochemical properties of the induced tumors appear to resemble the extensions of the gradual changes in some cases, but in others they are close to a maximum or minimum activity observed in the somewhat early period of the carcinogenesis. Which one of the above three types does the behavior belong to may also depend upon the kind of carcinogens, or upon the care of the animals including diet. The interpretation of the biochemical changes observed in the course of the carcinogenesis with respect to the mechanism of carcinogenesis has not been established satisfactorily in spite of a great deal of informations accumulated.

Since the chemical binding of carcinogenic aminoazo dyes with rats liver proteins in the precancerous stage was discovered by Miller et al. in 1947, it has been considered that this phenomenon may be closely related to the carcinogenic mechanism. Many studies, including those by the present authors have been reported with respect to the structure of the binding[8,9], mechanism of the binding[10,11,12], as well as the nature of proteins bound to the dye[13,14,15,16,17]. In the metabolism of the aminoazo benzene, the oxidative N-demethylation seems to be one of the most important reactions with respect to the dye-protein binding as well as the carcinogenic mechanism mainly from the following reasons: 1) 4-aminoazobenzene (AB) having no N-methyl group is not carcinogenic while dimethyl- or monomethyl-aminoazobenzene (DAB or MAB) are equally potent carcinogens for rats liver[18], and 2) Terayama et al.[12] recently observed the incorporation ofN-methyl carbon into a polar dye (Fr. 3) prepared from liver of rats fed monomethylaminoazobenzene containing ^{14}C-labelled methyl group.

The gradual decrease of the hepatic catalase activity of rats fed rice diet contai-

ning DAB has been reported since the early time of the carcinogenic study[19]. The catalase activity of the hepatoma has been reported to be very low[20]. Recently, Doi et al.[6], however, reported that the catalase activity reached a minimum value after the first one month of the DAB feeding and then gradually increased again and finally showed almost a similar activity to the normal liver. Mori et al.[21] reported that the catalase activity decreased continuously in the course of DAB feeding, but the activity was restored when the dye administration was discontinued. They imagined that the decrease in the catalase activity during the dye administration was due to the inhibitory effect by the dye or its metabolites still remaining in the liver. The present investigation is concerned partly with the elucidation of the peculiar behavior of the catalase activity. Besides catalase, N-demethylase, and xanthine oxidase have also been investigated, together with amount of protein bound dye, in the course of the repeated administrations of 3'-Me-DAB.

Experimental

Care of the Animals and the Administration of 3'-Me-DAB. Male albino rats weighing about 150g were fed the standard diet* for one week prior to the experiment. The same diet was given ad libitum throughout the experimental course. Generally once every seven days the rats were administered with 25 mg of 3'-Me-DAB dissolved in one ml. of corn oil** into stomach with the aid of a stomach tube. As controls, the equal volume of corn oil was administered. Two days after the last administration of the dye the animals were killed by decapitation and the liver was promptly perfused with physiological saline solution in situ. Then the liver was immediately removed and used for the assays. Usually two or more rats were sacrificed every time.

Estimation of the Protein-bound Dye. The defatted rat liver was hydrolysed with a protease (Pronase***) from *Streptomyces griseus*. The polar dye was extracted with n-butanol from the aqueous solution after the enzymic hydrolysis of the dye-bound protein (several days incubation at 37°C). The amount of the polar dye was estimated according to the procedure described by Kusama and Terayama[22] and tentatively expressed in term of DAB. The rest of the liver was used for the assay of the enzymes.

Measurement of N-Demethylase Activity. The hepatic N-demethylase activity against 3-Me-MAB as substrate was measured as follows: The liver (one to two g) was homogenized in a Potter-Elvehjem homogenizer in the presence of four times as much of a cold 1.15 per cent potassium chloride solution and used as the enzyme solution.

The composition of the reaction mixture was as follows (Table 1):

* Commercial product of the Oriental Yeast Industrial Company, Ltd, Tokyo, Japan.

** Product of Nagairyu Co., Ltd., Tokyo.

*** Kaken Kagaku, Co., Ltd., Tokyo.

Table 1. Composition of the Reaction Mixture for the Assay of N-demethylase

Componts	Concentration	Volume
TPN (Sigma Chem. Co.)	1 mg/ml	0.1 ml
DPN (Sigma Chem. Co.)	1 mg/ml	0.1 ml
Glucose-6-phosphate (Sigma Chem. Co.)	0.01 M	0.3 ml
$MgCl_2$	0.1 M	0.2 ml
KCl	0.2 M	0.2 ml
Nicotinamide	1 M	0.2 ml
Phosphate buffer (pH 7.4)	0.1 M	1.5 ml
Distilled water		0.8 ml
Homogenate	20 %	1.0 ml
3-Me-MAB (dissolved in ethanol)	0.1 %	0.1 ml
Total vol.		4.5 ml

The components mentioned in Table 1 were mixed in a small Erlenmeyer flask kept in ice. 3-Me-MAB, a suitable substrate[23] for the aminoazo dye N-demethylase, was added last and the flask was immediately transferred to a constant temperature bath regulated at 37°C. The flask was gently shaken for 30 minutes. The reaction was stopped by the addition of about 5 ml of acetone and benzene mixture (2 : 5 v/v), which simultaneously extracted the aminoazo dyes in the reaction system. The extractions were repeated with the same mixed solvent until the upper organic solvent layer separated by centrifugation became colorless. The extracts were combined and the solvent was removed by evaporation under the reduced pressure. The aminoazo dye present in the residue was dissolved in a small amount of petroleum ether and adsorbed at the top of a Brockmann's chromatographic alumina column (6×100 mm). By eluting with benzene, 3-Me-MAB, an original substrate, was separated first. 3-Me-AB, a demethylated product, was fractionated by eluting with a mixture of acetone and benzene (1 : 2). Both eluates were evaporated under reduced pressure. Each of the dyes was dissolved in 2N HCl-50 per cent ethanol mixture and the optical absorbance was measured at the wave length corresponding to the absorption maximum of each of the dyes, using a Beckman spectrophotometer. The demethylase activity was expressed in term of 3-Me-AB formed under the experimental conditions described above (see Fig. 2).

Measurement of Catalase Activity A part of the same homogenate used for the assay of N-demethylase activity was diluted tenfold with a phosphate buffer (pH 6.8, 0.05M) and used for the assay of the catalase activity. The modified Sumner and Somers's method[24] was employed. The composition of the reaction mixture is shown in Table 2. Every reaction component was kept at 0°C. The reaction was carried out in ice-water bath. At one, two, three and four minutes after the initiation of reaction, an aliquot of 5 ml each was quickly transferred to a flask (50 ml) containing

Table 2. Composition of Reaction Mixture for the Assay of Catalase

Components	Concentration	Volume
Phosphate buffer	0.05 M (pH 6.8)	50 ml
Hydrogen peroxide	Ca. 1 %	1 ml
Homogenate	2 %	0.2 ml

5 ml of 2 N H_2SO_4 in order to stop the reaction. The amount of H_2O_2 remaining was titrated with 0.005 N $KMnO_4$ solution. The $KMnO_4$ consumption at zero time was measured separately. The values of the velocity constants as a first order reaction were plotted against the reaction time and extrapolated to zero time. The initial velocity constant, Ks, was thus obtained. The catalase activity was defined as follows:

$$\text{Catalase Activity} = Ks/N \text{ (mg) in the homogenate used}$$

The nitrogen was estimated by the micro-Kjeldahl method.

Measurement of Xanthine Oxidase Activity Xanthine oxidase activity of liver of rats was measured according to Richert et al.[25] Two or three grams of fresh rat liver, added twice as much of water was homogenized in a Potter-Elvehjem homogenizer in the presence of a few drops of toluene at 0°C for four minutes. The homogenate was dialysed against tap water for about four hours, then against distilled water for 20 hours. Dialysed homogenate was used as the enzyme solution. The reaction for the assay of xanthine oxidase activity was carried out in Thunberg tube at 37°C and the decoloration time of methylene blue was measured. The composition of the reaction mixture is indicated in Table 3.

Table 3. Composition of Reaction Mixture for the Assay of Xanthine Oxidase

Thunberg tube:	0.5 M phosphate buffer (pH 7.5)	0.2 ml
	Enzyme solution	2.0 ml
Hallow stoyper:	0.5 % hypoxanthine in dil. KOH (dist. water for the control)	0.2 ml
	0.0113 M methylene blue	0.1 ml

According to the paper by Richert et al.[25], methylene blue decoloration time was cerrelated to the oxygene uptake measured by the manometric method. The comparison of the xanthine oxidase activity among normal liver, dye-fed liver, hepatic tumor, non-tumorous part around tumor was made. The activities of these pathological materials relative to the activity of the normal liver are illustrated in Fig. 4.

Results

Protein bound Dye As shown in Fig. 1 the amount of the polar dye reached a max. value after the first few administrations of the dye and then gradually decreased

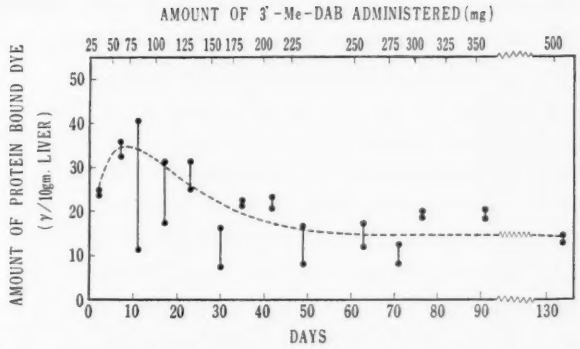

Fig. 1. Protein bound Dye (Polar Dye) Behavior in the course of the Repeated Administrations of 3'-Me-DAB.

even though the dye administration was repeated. After the tenth administration of the dye (amounting to 250 mg of the dye in total), the polar dye level became rather constant, keeping about 40 per cent of the max. level. This period coincides interestingly with that showing the recovery of N-demethylase and catalase. Possible mechanisms for the decreasing tendency of the polar dye after the prolonged administration of 3'-Me-DAB could be as follows: a) Loss of proteins responsible for the binding as suggested by Miller[18], and b) change in the metabolic activity of rat liver against the dye, i.e., the decrease in the metabolism responsible for the binding or increase of the antagonizing metabolism such as detoxication, excretion, etc. The latter possibility seems to be a most plausible one and the study is desired to be extended along this line.

N-Demethylase Activity The N-demethylase behavior in the course of the repeated administrations of 3'-Me-DAB is shown in Fig. 2. The activity decreased to the

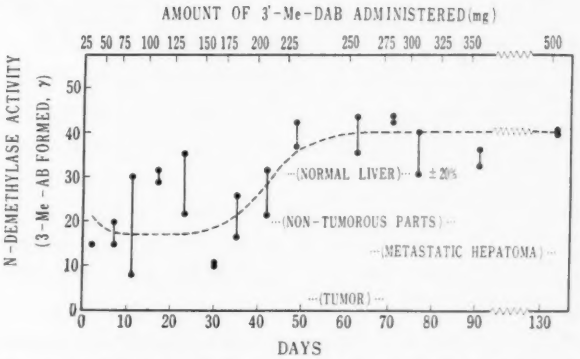
AMOUNT OF 3'-Me-DAB ADMINISTERED (mg)
25 50 75 100 125 150 175 200 225 250 275 300 325 350 500
N-DEMETHYLASE ACTIVITY
(3-Me-AB FORMED, γ)
0 10 20 30 40 50
(NORMAL LIVER) ±20%
(NON-TUMOROUS PARTS)
(METASTATIC HEPATOMA)
(TUMOR)
0 10 20 30 40 50 60 70 80 90 130
DAYS

Fig. 2. N-Demethylase Behavior in the course of the Repeated Administrations of 3'-Me-DAB.

lowest level even after a single does of 3′-Me-DAB, and apparently remained at about a half of the normal activity level up to the sixth administration, and then gradually recovered up to the normal or even a little higher level. The N-demethylase activity in the primary hepatic tumor which was generated by feeding 3′-Me-DAB -rice diet for three months was only a trace, but the activity of the non-tumorous part surrounding the tumor or the hepatoma infiltrated to the omentum was close to the minimum activity level, about 50 per cent of the normal activity.

The decrease in the enzymic activity in the early period of the dye administration must be due to the hepatic damage caused by the dye administered. The recovery of the enzymic activity shown after the repeated administrations of the dye suggests that the liver as a whole acquires a kind of resistance against the toxic effect of the dye administered or of the intermediate metabolites from the dye through the repeated administrations of the dye for a long period under good nutritional condition.

The very low enzymic level of the primary hepatoma[26] compared with the metastatic hepatoma may be partly be explained by the morphological defect (necrosis, change in the pattern of cellular species, etc.), even though only a peripheral part of the tumor was used in order to get rid of the necrotic tissue as much as possible, as well as by the actual loss of the enzyme in the tumor cells. The restricted supply of the nourishments due to the defect in the blood supply in the primary tumor compared with the normal tissue might also be a reason. The apparently non-tumorous part of the liver showed about a half of the normal activity level.

Catalase Activity As shown in Fig. 3, the catalase activity appeared to behave

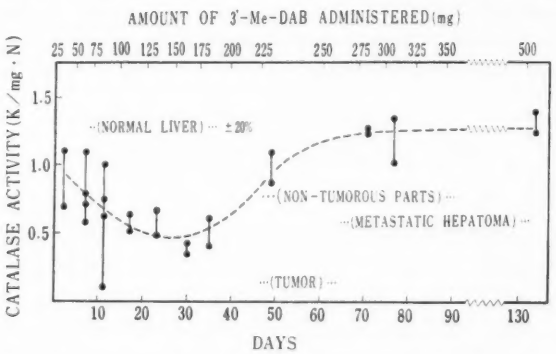

Fig. 3. Catalase Behavior in the course of the Repeated Administrations of 3′-Me-DAB.

in a similar way to N-demethylase, though the decrease in the early period was somewhat more gradual. The activity dropped down to the minimum level after the sixth administration of the dye and then gradually recovered up to the normal

Table 4. Catalase Activities of Liver of Rats with or without the Administration of 3′-Me-DAB and the Effect of Dialysis of Liver Homogenate upon Catalase Activity.

	Group: Treatment	Exp. No.	Catalase Activity	
			Before Dialysis	After Dialysis
I	Basal diet for 11 days	1	1.38	1.59
		2	1.14	1.36
II	Corn oil 1 ml. 5 days			
	corn oil 1 ml. 4 days	3	1.10	—
	corn oil 1 ml. 2 days	4	1.38	1.36
III	Dye 25 mg 5 days			
	dye 25 mg 4 days	5	1.02	0.96
	dye 25 mg 2 days	6	0.63	0.67

The dye was administered in a form of 1 ml. corn oil solution intragastrically by means of a stomach tube.

level after the tenth administration of the dye. The catalase activities of the primary hepatoma, hepatoma metastasized to the omentum, and the non-tumorous part of the liver around the hepatoma seemed to be similar to the behavior observed for the N-demethylase. The loss of the catalase activity in the early period was proved not to be due to the inhibition by the dye or the dye metabolites remaining in the liver as shown in the following experiments. The liver homogenate from rats administered three time with 3′-Me-DAB was dialysed against a phosphate buffer (pH 6.8, 0.05 M) at 2°C. The same catalase activity was observed between thus dialysed and non-dialysed liver homogenates (see Table 4). The decrease of the catalase activity in the first thirty days therefore must be due to the loss of the enzyme itself.

Xanthine Oxidase The results are summarized in Fig. 4. The xanthine oxidase activity of the liver decreased by the administration of the carcinogen as reported in the previous paper[27], using DAB instead of 3′-Me-DAB. The activity once decreased seems to recover, how. ever, after the repeated administrations. The hepatoma retained about a half of the normal hepatic activity, but the cholangioma showed almost null activity.

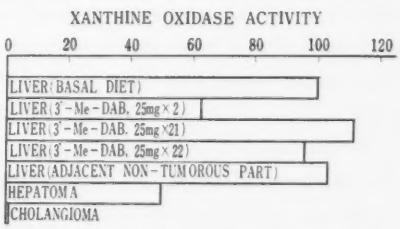

Fig. 4. Comparative Xanthine Oxidase Activities of Various Livers and Tumors.

Morphological Observation on the Specimen of Rats Liver Morphological examinations were carried out on the liver specimens of rats administered with 3′-Me-DAB.

1) 3′-Me-De-DAB, 25 mg x 2.

Degeneration and slight necrotic change were observed mainly at the central part of acini. Hyperplasia of cells in Glisson's capsule. Slight hyperplasia of bile duct cells also.

2) 3′-Me-DAB, 25 mg x 22.

Spreading out of the degenerative and necrotic region from the central part of acini. Hyperplasia of bile duct cells intra-acinally. Scattered presence of hepatic cells in mitosis, especially around the acini.

3) 3′-Me-DAB, 25 mg x 26, then eighty-eight days of the basal diet.

The structureof acini is similar to the normal. Slight and irregular necrotic layer and congestive layer are observed at the center and peripheral region of acini. Very slight hyperplastic layer is observed, especially around Glisson's capsule. The morphological observation does not predict the possibility of tumor genesis even if the rats were kept alive for longer periods.

Discussion

The present data demonstrated that some of the hepatic enzyme activities were greatly damaged by the administration of the carcinogenic aminoazo dye at the beginning, but in time such a hepatic damage became less and less in the course of the repeated administrations of the dye and finally the enzymic activities once dropped down to about fifty per cent of the normal level recovered again to the normal or a little higher one. The aquirement of the resistance against the toxic effect of the dye by rats liver may explain the observed events described here. The gastointestinal absorption of the dye was assumed not to be altered. The liver appeared to have gained resistance against the damage by the carcinogenic azo dye adaptively probably through the altered metabolism against the dye. It is very interesting to see that the activity level of some enzymes in the tumors are close to the minimal values observed in the course of the carcinogenesis. The similar observations are also reported with the swelling of mitochondria[28] or microsomes[29]. The initial acute hepatic damage may partly be ascribed to the formation and accumulation of the formaldehyde from N-methyl of aminoazo dye as described in the case of dimethylnitrosamine[30][31].

The biochemical properties of the liver as a whole are the average pattern. All the cells of the liver do not respond to the effect by the carcinogen in the same way. The sensitive cells are damaged more severely by the carcinogen and often they die. The death of the old, differentiated but non-resistant hepatic cells may be succeeded by the young, immature but somewhat resistant cells by the regeneration process. The stage of the enzymic recovery after passing the minimum level may correspond to the stage of this regeneration. Throughout the early period of

the dye administration, the death and the regeneration of the cells are repeated and in time the liver acquires more and more resistance against the dye and finally most of the hepatic cells become rather insensitive to the toxic effect of the dye. The enzymic level of liver as a whole recovers fully at last. Same precancerous cells which have lost some enzymic activity genotypically (or irreversibly) could be generated and the transformation could further be pushed forwards to the more advanced malignancy if this tumultuous period under the abnormal biochemical and histological circumstances is prolonged.

Under the experimental conditions described here, it is clear that the hepatic damage does not go too far and soon the liver becomes adapted to the repeated administration of the dye and insensitive to the injury. The morphological lesion of the liver of rats therefore was rather slight even after the twenty-sixth administration of 3′-Me-DAB (650 mg in total) and no incidence of hepatoma was observed even after two and a half months following the twenty-sixth administration of the dye. This amount (650 mg in total) is large enough to produce one hundred per cent incidence of hepatoma under the usual carcinogenic, experimental condition, i.e., 0.06 per cent dye-containing rice as a basal diet being fed.

Summary

25 mg of 3′-Me-DAB dissolved in one ml of corn oil was administered intermittently (usually once a week) to rats for a long period with a help of a stomach tube. Two days after the last administration of the dye, the liver was removed and the protein bound dye, N-demethylase, catalase and xanthine oxidase activities of the liver were measured. The results are summarized as follows:

1) The amount of polar dye reaches a maximum after a few administrations of the dye, then gradually decreases and finally levels off.

2) The catalase activity decreases at first, reaching a minimum, and then gradually recovers to the normal level. The decrease of the catalase in the early period does not seem to be the result of the inhibition by the dye or its metabolite, but due to the lose of the enzyme itself.

3) The N-demethylase and xanthine oxidase seem to behave similarly to the catalase during the course of the dye administration.

4) Then the interpretation for the enzymic behavior observed during the repeated dye administrations was suggested. After the acute toxicological damage period at the beginning, the hepatic cells survived may regenerate, and the regenerated young cells become more and more resistant to the damaging effect by the carcinogen probably through the altered metabolic activity against the carcinogenic aminoazo dye. Finally the liver becomes completely resistant and no more hepatic damage is

produced even by the repeated administrations of the dye, so far as the biochemical analysis indicates.

Under the condition described herewith, the hepatic damage does not go far enough and the chance for the formation of the neoplastic cells seems not to be great.

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[GANN, 51, 265~271; September, 1960]

THE INFLUENCE OF NICOTINAMIDE AND DIPHOSPHOPYRIDINE NUCLEOTIDE ON AZO DYE AND METHYLCHOLANTHRENE CARCINOGENESIS

(With Plates XIX and XX)

MUTSUSHI MATUYAMA and TAKEO NAGAYO
(Department of Pathology, Nagoya City University School of Medicine)

It has been established by many workers that the pyridine nucleotide (PN) content of tumour is generally very low as compared with that of normal tissue (1-10). That nuclear diphosphopyridine nucleotide (DPN) pyrophosphorylase activity limiting the DPN synthesis is significantly reduced in mammary gland carcinomata has also been reported (8). On the other hand, it was reported (11, 12) that a marked increase of DPN content occurred in the liver and transplanted tumours following the injection of nicotinamide and other related substances into mice.

Recently, Oide (13) found that when a pellet of nicotinamide is intrasplenically implanted in rats after partial removal of the liver, the DPN content of regenerating liver is invariably increased after 24 hours, and at the same time the number of mitosis is greatly reduced. Further, he (14) showed that the increase of DPN level by nicotinamide administration is very low in the Ehrich ascites tumour cell and the liver of its host mouse, as compared with that of normal liver.

Fujii and Mizuno (15) demonstrated that nicotinamide has a marked inhibitory effect on the epidermal hyperplasia induced by methylcholanthrene (MC) painting. These observations seem to suggest that the rate of cell division is closely associated with the DPN content of the cell, whether normal or tumour cells. However, so far as is known, there is no direct evidence as to whether the nicotinamide injection can retard the development of experimental tumour. Therefore, it was attempted in this study to examine whether nicotinamide can inhibit the incidence and growth of tumours induced by two potent carcinogens.

One experiment was designed to investigate the effect of this substance on the p-dimethylaminoazobenzene (DAB) carcinogenesis, and another on the MC tumourigenesis. Furthermore the effect of DPN on the MC tumourigenesis was also examined.

Materials and Methods

Experiment using DAB as carcinogen.——A total of 55 rats of Sprague-Dawley

strain (from a city dealer) were used. These were divided into the following three groups:

Group 1: Fifteen rats were placed on basal diet (rice grains) and injected subcutaneously with 0.25 ml of 1 per cent nicotinamide*.

Group 2: Fifteen rats were placed on DAB diet and injected with 0.25 ml of cold sterile distilled water.

Group 3: Twenty-five rats were placed on DAB diet and injected subcutaneously with equal volume of 1 per cent nicotinamide solution.

The basal diet consisted of unpolished rice containing about 2.7 mg riboflavin per kg and about 75 g protein per kg. The amounts of basal diet consumed totaled 550-850 g per rats. DAB** was administrated following the method employed by other investigators. Namely, it was dissolved in olive oil, and solution was evenly mixed with unpolished rice, and the rats were fed on this mixture. The rats of DAB feeding groups were fed in the first week with a diet containing 0.02 per cent DAB, in the second week with that containing 0.04 per cent, and thereafter with a diet containing 0.06 per cent. Some green vegetables were given daily. Nicotinamide was dissolved in cold distilled water. Injections of three groups were started 1 week before commencement of azo dye feeding, and continued 3 times weekly. All rats were killed under ether narcosis when they fell into agonized condition, and a careful autopsy was performed. This experiment was terminated about 160 days after commencement by killing the surviving rats.

The tumours, liver and the metastatic tumour nodules were subjected to a histological study. These tissues were fixed with 10 per cent formalin, and sections were stained with hematoxylin and eosin.

Experiment using MC as carcinogen.——A total of 36 Wister strain rats weighing around 125 g were used. These were divided into three groups, each of which consisted of 12 animals; Rats in each group received subcutaneous injection of 0.25 ml of cold sterile distilled water (group 1), 1 per cent nicotinamide (group 2) or 0.1 per cent DPN*** (group 3) respectively. Solutions of nicotinamide and DPN were prepared in the same manner as in the first experiment. These injections were performed on the back of rat 3 times a week till the animals died or were killed. One week after the first injection, all rats were injected subcutaneously in intrascapular region with 3 mg of MC**** in 0.4 ml of olive oil. The rats were maintained on the usual laboratory diet of whole weat grains with daily supply of some green vegetables. After the first detection of tumour, the presence of tumours

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*, ** The sample of nicotinamide and DAB was kindly supplied by the Katayama Chemical Works, Japan.

, * The sample of DPN used was obtained from the Nutritional Biochemicals Corporation (N. B. C.), U. S. A. and MC was a product of the L. Light and Co., Ltd., England.

were examined by palpation twice a week. Autopsy and histological examination were performed in the same manner as in the first experiment.

Experimental Results

Effect of nicotinamide on the liver cancer induced by DAB.——Throughout the whole groups, the first tumour was detected 109 days after commencement of DAB feeding. Out of the 55 rats, 35 rats survived the injection period of over 100 days, receiving over 40 injections. Twenty rats died of severe pneumonia and lung abscess within 100 days after the begining of the injections. All animals which were fed with dye diet and survived the experimental period over 100 days (25 rats of group 2 and 3) developed hepatoma (Fig. 1). The general records of this experiment were summerized in Table 1. The average liver weight and index of the animals

Table 1. The effect of nicotinamide on the experimental production of liver cancer. Figures in parentheses represent the range in each group.

Group		Rice diet and nicotinamide inj.	DAB diet and water inj.	DAB diet and nicotinamide inj.
No. of rats at initial		15	15	25
No. of rats which survived for more than 100 days after first injection		10	12	13
Average body weight (g)	at initial	100	99	105
	at final	152	121	112
Survival in days		(all 161*)	(142-160*)	(114-160*)
Average liver weight (g) and index**		6.2 4.1	29.2 24.0	26.1 22.7
Average spleen index**		0.4	1.7	2.1
Amounts of nicotinamide injected (mg)		(all 162.5)	(−)	(112.5-162.5)
Amounts of DAB ingested (mg)		(−)	(473-534)	(327-557)
Liver findings***	−	10		
	±			
	+		1	4
	++		4	4
	+++		7	5

* This experiment was terminated 160 to 161 days after commencement.

** Index of liver or spleen is calculated by following equation:

$$\text{Liver (Spleen) index} = \frac{\text{Liver (Spleen) weight}}{\text{Body weight at final}} \times 100$$

*** −·····indicates smooth, practically normal liver;
±·····indicates nodular cirrhosis with adenomatous hyperplasia;
+·····indicates distinct area of cholangioma or hepatoma or both;
++·····indicates extensive liver cancer without metastasis;
+++··· indicates extensive liver cancer with metastasis (Sugiura, 1948).

of azo dye feeding groups were; 29.2 and 24.0 in group 2, 26.1 and 22.7 in group 3, respectively. These values indicate that no difinite difference exists between the two groups of azo dye feeding. But in the animals of the nicotinamide and DAB group (group 3), liver tumours were found to be more circumscribed than those in the animals of DAB group (group 2). In the control animals, the induced liver tumour reached a great size and often metastases appeared in the abdominal lymph nodes and peritoneum (Fig. 4).

Histologically, the liver tumours in the animals of DAB feeding groups were malignant liver cancer and revealed various deviations, for example, consisted of solid (Fig. 2), alveolar, trabecular and adenomatous (Fig. 3) types, or otherwise was accompanied often by bleeding and necrosis. Therefore, there were scarcely demonstrable differences between the DAB group and DAB plus nicotinamide group. But, the liver cancers induced in the nicotinamide plus DAB group were mainly an adenomatous type of hepatoma accompanied by increase in connective tissue. The livers of the nicotinamide group (group 1) showed no remarkable change macroscopically, but microscopical examination revealed that the parenchymal cells of these were considerably atrophic and degenerative.

Effects of nicotinamide and DPN on the MC sarcoma.——During the course of this experiment, all animals showed gradual increase in weight except 3 rats which died of sever pneumonia and lung abscess without any tumour within 100 days after

Table 2. The effect of nicotinamide and DPN on the incidence and growth of MC sarcoma.
Figures in parentheses represent the range in each group.

Group		MC and Water	MC and nicotinamide	MC and DPN
No. of rats at initial		12	12	12
No. of rats which survived for more than 100 days after MC injection		12	10	11
Average body weight (g)	at initial	131	121	123
	at final	174	184	167
Days when tumour found		(96–120)	(92–168)	(98–more than 168)
Average liver index*		3.5	4.7	4.6
Average spleen index*		0.27	0.35	0.34
Average tumour size (maximal diameter) (cm)		6.2	5.3	4.1
Average tumour weight (g) and index*		48.1 28.1	34.9 17.8	27.6 15.7
Amounts of compounds injected (mg)		(−)	(117.5–187.5)	(14.25–18.75)

* Index of liver, spleen or tumour is calculated in the same manner in Table 1.

injection of MC. The remaining 33 rats survived more than 100 days after the injection of MC, receiving over 47 injections of water, nicotinamide or DPN. The general records of this experiment were summerized in Table 2. Tumour nodule was first palpated after 92 days after injection of MC at the site of injection and 145 days after injection almost all the animals showed tumour nodules. Twenty-three of tumour-bearing animals died within 167 days, and remaining 10 animals were sacrificed on the 168 days after the MC injection. The time of "50 and 100 per cent tumour incidence" in each group was; 101st and 120th day in group 1, 122nd and 168th day in group 2, and 124th and more than 161th day in group 3. Furthermore, the tumour-incidence rates in groups 2 and 3 were still 30 and 36 per cent respectively, even when that in group 1 reached 100 per cent (Chart 1). From these results, it may be said that the incidence of MC sarcoma was retarded by 3 to 6 weeks in the groups of nicotinamide or DPN injection, compared with that in the group of water injection. Average values of tumour weight and tumour index on each group were as follows, 48.1 g and 28.1 in group 1, 34.9 g and 17.8 in group 2, and also 27.6 g and 15.7 in group 3 (Table 2). These results denote that nicotinamide and DPN retarded also the growth of tumour.

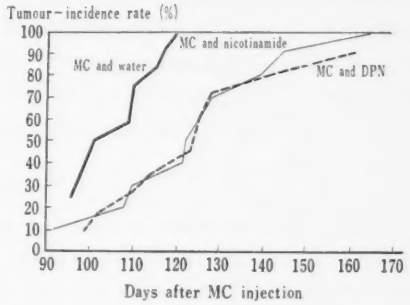

Chart 1. Inhibiting effect of nicotinamide and DPN on the incidence of MC sarcoma in rats.

Besides, the liver and spleen were atrophic in many cases of control group. All induced tumours were fibrosarcomas (Fig. 7), and no difference was observed among three groups. Attempts to transplant the tumour to normal Wister strain rats were made using the tumor of group 2. This tumour was highly transplantable so far as tried.

Discussion

From the results of second experiment, it is evident that nicotinamide has a inhibitory action on the MC tumorigenesis in rat. That the presence of nicotinamide greatly inhibits cozymase nucleosidase (DPNase) in rat brain and in rat liver has been reported by Mann and Quastel (16). Further, Oide (13) found that the DPN content of rat liver is increased by administration of nicotinamide, and at the same time the number of mitosis of rat liver is reduced. Therefore, it may be probable that nicotinamide becomes accumulated in the cytoplasm and so exerts its influence by inhibiting the cell division. It is also conceivable that nicotinamide serves as a

precursor of nicotinamide mononucleotide, which is essential for the synthesis of DPN (17). From this conception, the possibility that nicotinamide enhances the rate of nuclear DPN synthesis and increased DPN may results in inhibition of cell division cannot be excluded. But nicotinamide did not show a clear cut inhibition upon DAB liver cancer. The question that arises in the present experiments is the difference in the effect of same compound on the carcinogenesis of two different carcinogens.

Mann and Quastel (16) also reported that DPNase occured in rat liver and was apparently absent in dialysed rabbit skeletal muscle extract. If the DPNase in rat connective tissue is also absent as that in rabbit skeletal muscle, the findings of Mann and Quastel seem to be very attractive in explaining the above mentioned question. Because it is expected that in rat connective tissue, in which the destruction of DPN may hardly operate, DPN accumulation induced by nicotinamide injection may be greater than that in rat liver, and that antimitotic effect of nicotinamide in the former may be more prominent than that in the latter.

Summary

Using rats, the authors studied the effects of nicotinamide upon the incidence and growth of experimental tumours induced by p-dimethylaminoazobenzene (DAB) and 20-methylcholanthrene (MC). The effect of diphosphopyridine nucleotide (DPN) on the incidence of MC sarcoma was also examined additionally. The incidence and growth of MC sarcoma was delayed by administration of nicotinamide and DPN. In the case of DAB liver cancer, however, the inhibitory effect was very slight. The difference in the effect of nicotinamide on the tumourigenesis of two different carcinogens was discussed.

Acknowledgments

We wish to express our appreciation to Ass. Prof. I. Hirono and Dr. S. Iwase, the 2nd Department of Pathology, Nagoya University School of Medicine, for their keen interest in this study and for going through this paper before its publication. Our thanks are also due to Mr. Y. Kobayashi, Mr. J. Kiyono, Mr. T. Mazima, Mr. A. Maekawa, Mr. K. Uemura, and Mrs. C. Matsuyama of our laboratory for their technical help.

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Explanation of Plates XIX and XX

Fig. 1. All livers. Left 2 rows (10 samples) represent those of nicotinamide group, middle 3 rows (12 samples) those of DAB growp, and others (13 samples) those of nicotinamide and DAB group.

Fig. 2. Histological picture of liver tumour of DAB group, ×600. Cancer cells proliferated irregulary in the fibrous stroma. This is what is called the solid type.

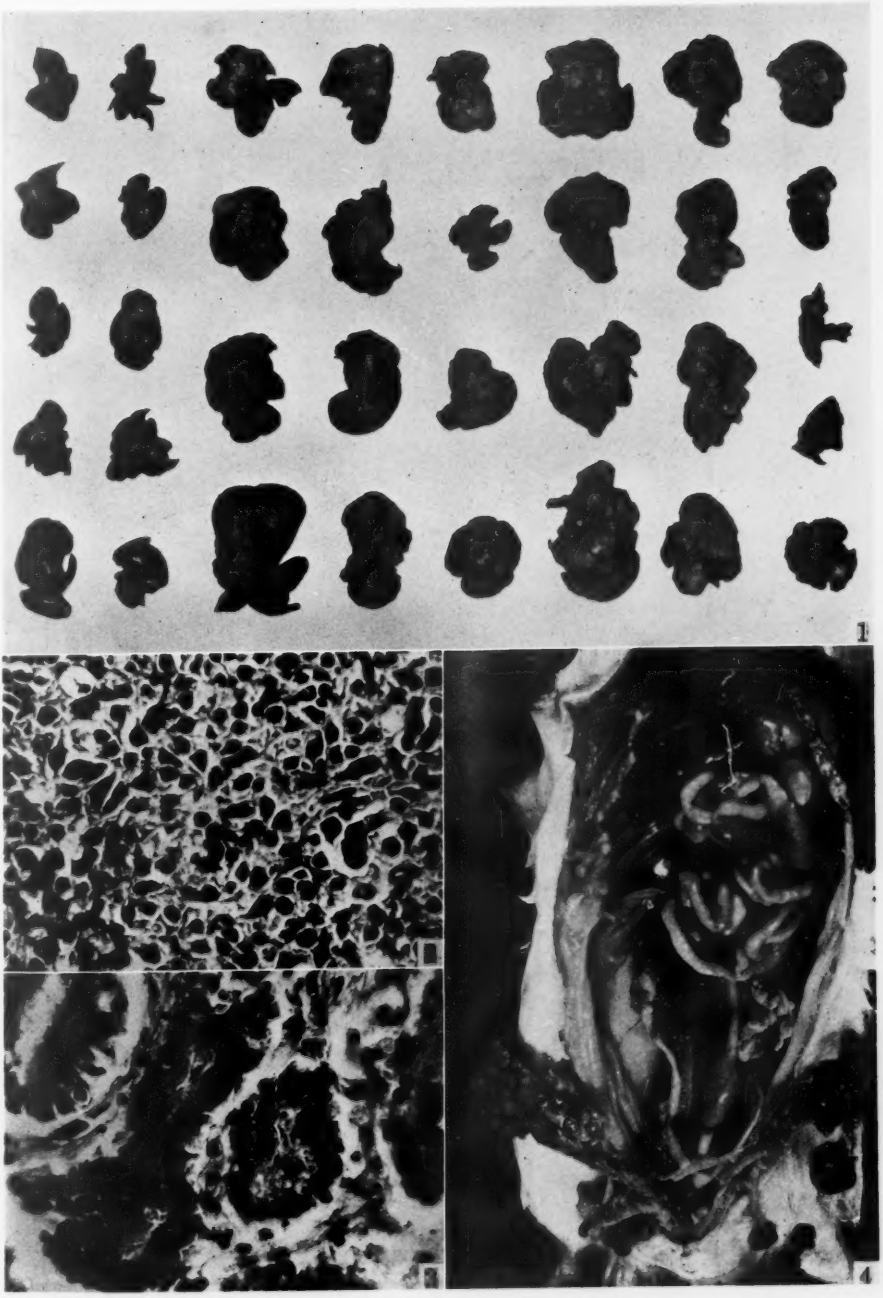
Fig. 3. Histological picture of liver tumour of nicotinamide and DAB group, ×600. Marked adenomatous structures of cancer cells, provided with connective fibers as stroma. This belongs to cancer of adenomatous type.

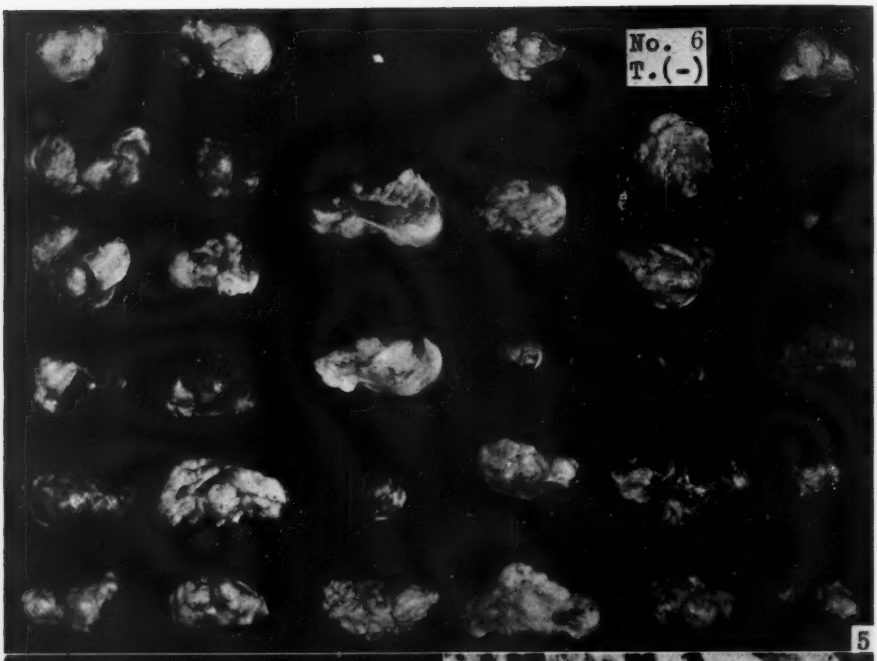
Fig. 4. Metastatic nodules of rat of DAB group. Note the soy-bean root like nodules of peritoneum. These are black in color due to haemorrhage.

Fig. 5. All MC sarcomas. Left 2 rows (12 samples) those of control group, middle 2 rows (10 samples) those of nicotinamide group, and others (11 samples) those of DPN group.

Fig. 6. Rat of control group bearing MC sarcoma which consists of apparently two nodules.

Fig. 7. Histological picture of the tumour shown in Fig. 6, ×800. Giant cells are numerous in this figure.

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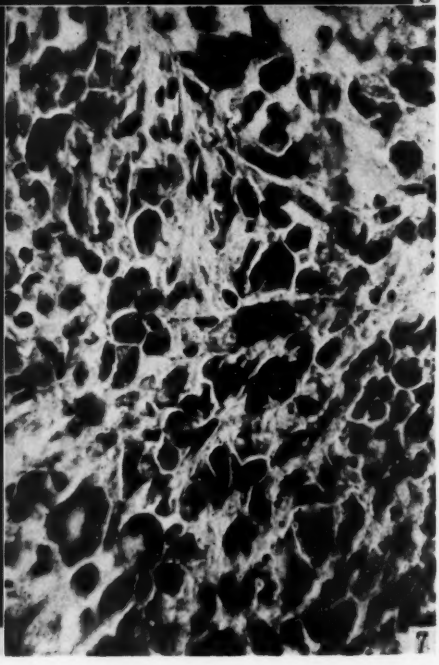
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[GANN, 51, 273~284; September, 1960]

ON THE AGE FACTOR IN THE HISTOLOGICAL APPEARANCE AND ITS VARIATION IN GASTRIC CANCER

(With Plate XXI)

HISASHI TAUCHI, TSUNEKO SATO, HISANDO KOBAYASHI and WATARU KAWABE

(The 2nd Department of Pathology, Nagoya University School of Medicine
Director, Prof. H. TAUCHI, Nagoya)

The histological appearances of the cancer are very varied due to the protean morphology of the cancer cells, the varied attitudes the interstitium presents, the multiferous relation existing between the cancer cells and interstitium, besides the basic characteristics of the cancer cells multifying ad infinitum. Tauchi et al. and Sato reported on the mechanism of the variation of the cancer histology examining many cancer tissues originated from many organs,[8)29)30)31)32)40)41)42)43)48)] but there are many problems yet to be clarified.

The histological picture of the cancer tissue is also varied according to age of the host[15)21)23)34)35)47)], and in this variation there are some problems that need to be made clear.

The present authors have conducted studies on the varied histological picture of gastric cancer especially in relation to the age of the host, and some interesting results obtained will be presented and discussed here.

Materials and Methods

Three hundred and seven cases of gastric cancer were employed, 228 surgical, and 79 autopsy cases. These cancer patients were from 16 to 79 years old, and 187 cases of them were males and 120 were females. Besides, 7 autapsy cases of gastric cancer were also examined where the cancer tissues were very small in extent and discovered incidentally at autopsy.

All these cancer tissues were fixed in formalin as usual and many sections were made from the primary and metastatic foci. Paraffin sections were stained mainly with hematoxylin and eosin, but when necessary the silver impregnation of Bielschowsky, periodic acid-schiff stain after Lillie, mucicarmine stain and v. Gieson and Weigert stains for elastic fibers were employed.

The histological pictures of the cancer tissues were classified according to the authors' methods.[43)] According to the histological character, the interstitium of the

cancer tissues was classified into 5 types, namely, fibrillar, loosely fibrous, fibrous, hyaline and mucous interstitia.

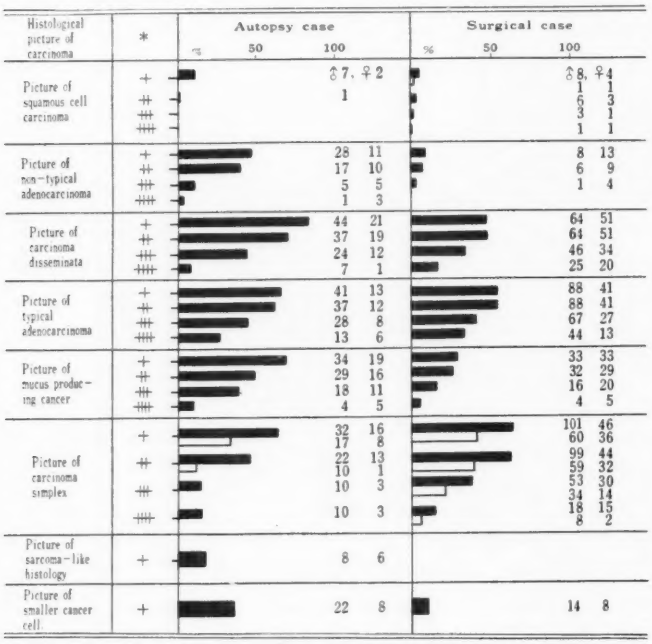
The histological appearances of cancer parenchyma and interstitia of many areas of the cancer tissues were compared with each other. And ranges and frequencies of appearance of various histological pictures were compared between the surgical and autopsy cases and according to age and sex of the host. The pattern of relationship between the character of cancer parenchyma and of interstitia was also compared between the various areas of cancer tissues and cases of the disease.

Results

Results obtained are summarized and presented in Fig. 1~Fig. 4.

Fig. 1 Comparison of Range and Frequency of Appearance of Histological Picture of Stomach Cancer.

(Number of respective cases are indicated as percentage of total cases examined)
(Number of male or female cases are indicated respectively by ♂ and ♀)



[ ] indicates a picture of basalioma in case of squamous celled carcinoma, and a picture of lumina formation in case of carcinoma simplex.

*+ Cases with picture of respective cancer histology.

++ Cases with picture of respective cancer histology extending over more than 1/10 of cancer mass.

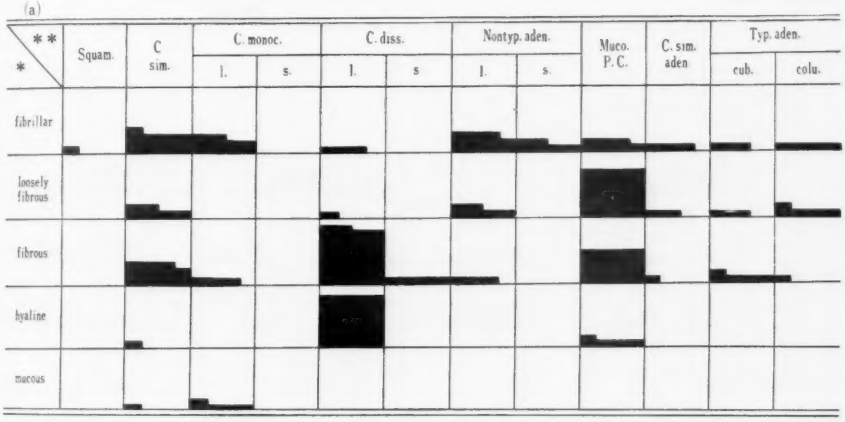
+++ Cases with picture of respective cancer histology extending over more than 1/4 of the cancer mass.

++++ Cases with picture of respective cancer histology extending over more than 1/2 of of the cancer mass.

Fig. 2 Relationship between Grade of Appearance of Histological Pictures of the Cancer Parenchym and of the Interstitium in Each Decade.

a) In case of age under 39 years.
b) In case of age from 40 to 49 years.
c) In case of age from 50 to 59 years.
d) In case of age from 60 to 69 years.
e) In case of age above 70 years.
* Histological picture of cancer stroma.
** Histological picture of cancer parenchym.
Abbreviations:
Squam.=Squamous cell carcinoma, C. Sim.=Carcinoma simplex, C. Monoc.=Carcinoma monocellulare, l.=large cancer cells, s.=small cancer cells, C. diss.=Carcinoma disseminata, Non-typ. Ade.=Non-typical adenocarcinoma, Muco. P.=Mucous producing cancer, C. Sim. aden.=Carcinoma simplex with lumina formation, Typ. ade.=Typical adenocarcinoma, cub.=cuboidal cell, colu.=columnar cell.

(a)



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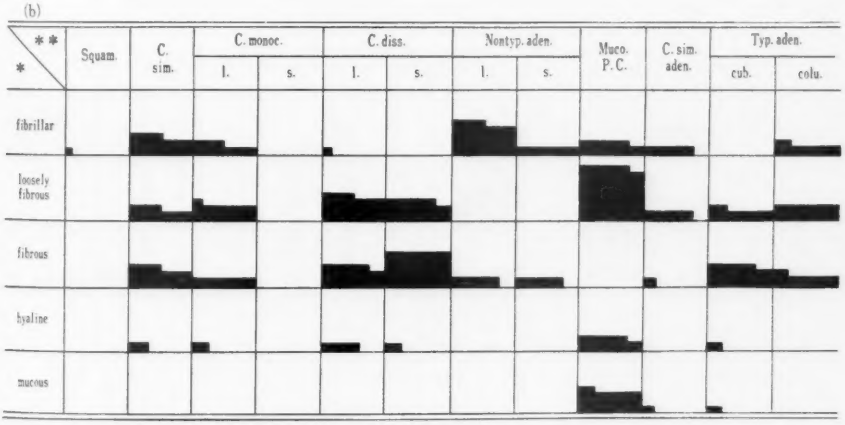

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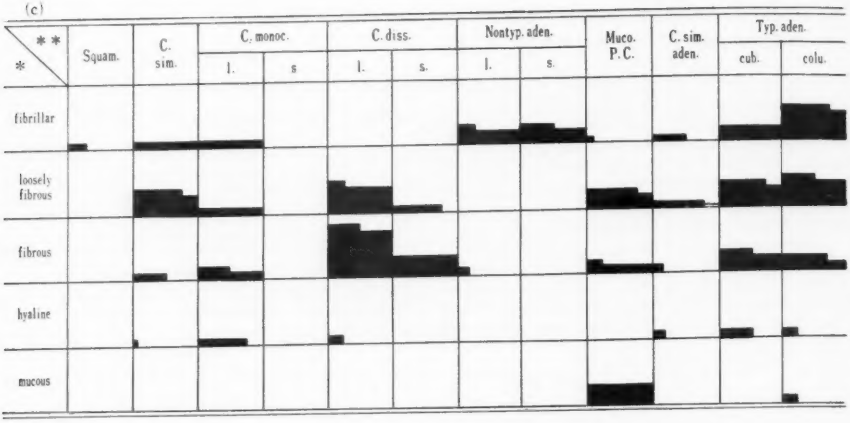
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hyaline
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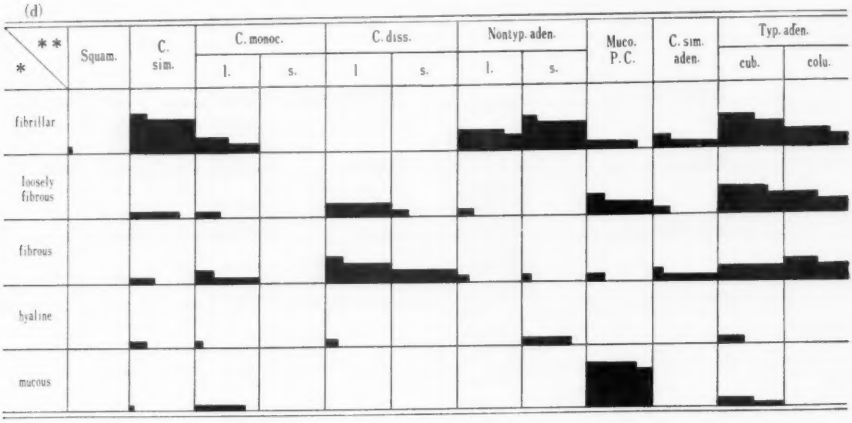
(d)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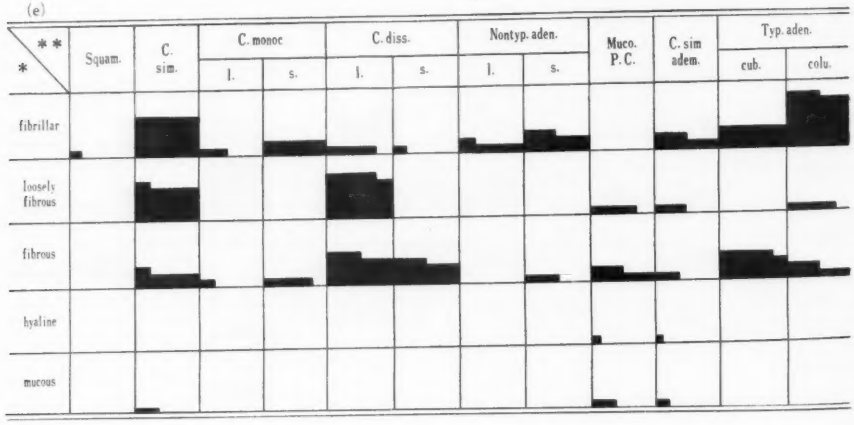
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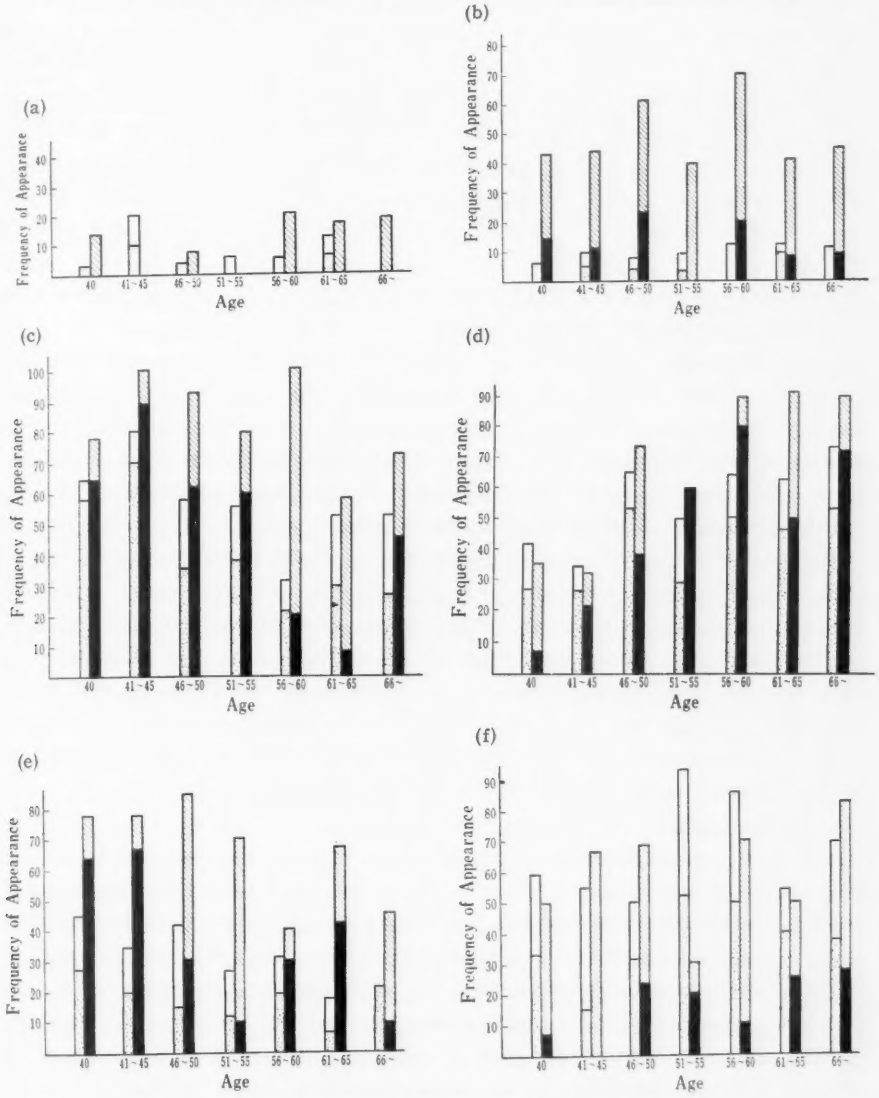
Fig. 3 The Comparison of Frequency of Appearance of Histological pictures according to Age.
(Number of respective case are indicated as percentage in total cases in each age group)



Cases (%) with a picture extending over less than 1/4 of the cancer mass in operation case.
Cases (%) with a picture extending over more than 1/4 of the cancer mass in operation case.
Cases (%) with a picture extending over less than 1/4 of the cancer mass in autopsy case.
Cases (%) with a picture extending over more 1/4 of the cancer mass in autopsy case.

In Fig. 3:

a) Picture of squamous cell carcinoma.
b) Picture of non-typical adenocarcinoma.
c) Picture of carcinoma disseminata.
d) Picture of typical adenocarcinoma.
e) Picture of mucous producing cancer.
f) Picture of carcinoma simplex.

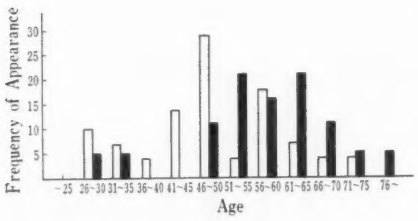


Fig. 4 Comparison of Frequency of Appearance of Pictures of Non-Typical Adenocarcinoma according to Age in Autopsy Cases of Stomach Cancer.

□ Cases (%) with picture of non-typical adenocarcinoma consisting of larger cancer cells.
■ Cases (%) with picture of non-typical adenocarcinoma consisting of smaller cells.

1) **On the picture of epidermoid cancer**

In surgical cases, the pictures of epidermoid cancer were seen in only a few cases, where these pictures were seen in relatively wider range. On the contrary, in autopsy cases, the pictures were seen more often but in narrower range. The pictures of epidermoid cancer were also noticed more frequently in older autopsy cases. The interstitia of epidermoid cancer were fibrillar in almost all the cases.

2) **On the picture of non-typical adenocarcinoma**

The pictures of non-typical adenocarcinoma were seen more frequently and in wider range in autopsy cases than in surgical cases, and in the latter cases they were seen in narrower range only in a few cases. And it was noticeable that many of the autopsy cases with that picture in wider range and of the surgical cases with that picture were female cases. In autopsy cases, the cancer cells of this carcinoma type were frequently small in size in older age. The interstitia of non-typical adenocarcinoma were frequently fibrillar. And the histological picture of the interstitia was not influenced by the age difference of the host.

3) **On the picture of carcinoma disseminata**

In autopsy cases, the picture of carcinoma disseminata was generally more frequently seen and the range of its appearance in each case was also generally wider. However, it was noticeable that cases with the picture of the cancer extending over more than half of the cancer mass were found more frequently in surgical cases than in autopsy ones. The interstitia of carcinoma disseminata were most frequently fibrous, sometimes loosely fibrous, and less frequently hyaline or fibrillar. Hyaline interstitia were found only in the cases of the age under 39 years. In autopsy cases, the interstitia of carcinoma disseminata had a tendency to become generally softer with advancing age of the host. The interstitia of the cancer tissue formed of small cancer cells were rather sclerotic. Surgical cases with the picture of carcinoma disseminata in wider range were of rather younger age, in contrast to

the cases with the picture of typical adenocarcinoma.

4) **On the picture of typical adenocarcinoma**

Cases with the picture of typical adenocarcinoma were somewhat frequently seen in autopsy cases, but cases with that picture in wider range were more frequently seen in surgical cases. The frequency of cases with the picture of typical adenocarcinoma was compared between each decade, in order to examine the pattern of distribution of the cancer histology according to age. Surgical cases with this picture became somewhat increased in percentage with advancement of age, but autopsy cases with this picture became remarkably increased in percentage with advancement of age. This tendency was more marked in cases where this cancer histology was found in wide range.

In autopsy cases, the interstitia of typical adenocarcinoma especially made of high columnar cancer cells were generally fibrillar, sometimes loosely fibrous. Fibrous interstitia were only rarely found in the picture of typical adenocarcinoma made rather of cuboid cells than columnar ones.

5) **On the picture of mucous producing cancer**

Generally speaking, the picture of mucous-producing cancer was more frequent in autopsy cases than in surgical cases. The frequency of cases with the picture of mucous-producing cancer was compared between each decade in order to examine the pattern of distribution of this cancer histology according to age. Contrasting to the case of typical adenocarcinoma, surgical cases with the picture of mucous-producing cancer became somewhat decreased in percentage with advancement of age, and autopsy cases with this picture became remarkably decreased in percentage with advancement of age. This tendency was more marked in cases where this cancer histology was found in wider range.

In autopsy cases, the interstitia of mucous-producing cancer were mucous, loosely fibrous or fibrous.. In case of this type of histology, the picture of emancipated infiltration of cancer cells was frequently seen especially in cases of younger age, where the interstitia had a tendency to proliferate more vigorously.

6) **On the picture of carcinoma simplex**

The frequency of the picture of carcinoma simplex was not different between surgical and autopsy cases, and as far as ages of the hosts are concerned, cases with this cancer histology were distributed in all decades more widely in autopsy cases than in surgical cases.

Interstitia of carcinoma simplex, in autopsy cases, were rather frequently fibrillar or loosely fibrous, and became more fibrillar with advancing age.

7) **On the transition between the pictures of various cancer histology**

Between the above mentioned types of cancer histology, there were often noticed transitional pictures from one to the other.

8) **On the autopsy cases with a small focus of gastric cancer tissue**

The average age of 7 autopsy cases, where a small focus of the cancer was incidentally found at autopsy, was 62 years, which was far older than average age of all the autopsy cases presented here. In these 7 autopsy cases, cancer tissues showed mostly a picture of typical adenocarcinoma, sometimes a picture of mucous-producing cancer but no picture of non-typical adenocarcinoma.

Summary and Discussion

Tauchi et al[43] and Sato[31] had investigated the histological pictures of cancer tissues examining the difference of histological pictures between autopsy cases and surgical ones, and presented some interesting conclusions as follows:

The histological picture of cancer tissue was generally relatively simple in the early stage of the disease but it became varied in the later stage, because various kinds of pictures of cancer were derived from the original cancer histology in the course of its growth. And they emphasized that influence of tissue environment may play an important part in the variation of cancer histology. The present authors have studied in the same way the histology of much larger number of cases of gastric cancer, and have found that even in case of gastric cancer alone, cancer histology became more varied in the course of its growth, and also that there were noticed some interesting findings according to age of the host in the picture of cancer histology and also in the pattern of combination of parenchyma and interstitia.

1) **On the pattern of appearance of some cancer histology according to age**

Pictures of typical adenocarcinoma were more frequently seen in older age, while, on the contrary, those of mucous producing cancer were more often seen in younger age. This tendency was much more marked in autopsy cases than in surgical cases.

Tauchi had previously examined[37] the cancer histology in cases of younger age and suggested that the cause of gastric cancer in younger age might be different from that of general gastric cancer. Nagayo[21] considered that the typical adenocarcinoma originated from the site of metaplastic gastritis and that the latter picture was frequently seen in older age. Oota[24][25] and Mulligan[20] also reported that the difference in cancer histology was due to the difference of its matrix tissue.

However, in the pattern of appearances of pictures of typical adenocarcinoma and mucous-producing cancer according to age, significant differences have been found, by the present authors, between autopsy and surgical cases. Namely, in surgical cases, which may be considered as earlier stage of the disease, the difference in the frequency between these cancer pictures according to age was less marked, while on the contrary, in autopsy cases, considered as representing later stage, the difference according to age was more marked.

This fact suggests that the changing tendency from a picture of typical adenocar-

cinoma seen in earlier stage into a picture of mucous-producing cancer in the course of growth of cancer tissues is more marked in younger age, or that the changing tendency from a picture of mucous-producing cancer into a picture of typical adenocarcinoma in the course of cancer growth is more marked in older age.

These changes of pictures between typical adenocarcinoma and those of mucous-producing cancer are considered, on the one hand, to take place through transitions between those cancer cells themselves, and on the other hand, to take place for the reason that the mucous-producing cancer cells grow more rapidly than typical adenocarcinoma cells in younger age.

Pictures of typical adenocarcinoma with high columnar cells and of squamous celled cancer were more frequently seen in older age, while, on the contrary, pictures of mucous-producing cancer and of carcinoma disseminata were frequently seen in younger age. This tendency was more marked in autopsy cases than in surgical cases. In other words, the cancer cells generally were considered to have a tendency in the course of growth to become more differentiated in older age and more undifferentiated in younger age.

2) **On the pattern of interstitia of cancer tissues according to age**

The present authors pointed out that the interstitium was more frequently fibrillar in older age and more often fibrous in younger aged. There was also found somewhat close relationship between the histological picture of parenchyma and of interstitia. However, interstitia in cases of the pictures of typical adenocarcinoma composed of high columnar cells and of squamous celled cancer, which pictures were often seen in older age, were frequently fibrillar, and proliferation of interstitia in case of carcinoma disseminata and simplex was less marked in older age than in the younger.

One of us, Takeuchi[36], had found the interesting differences in the distribution of acid mucopolysaccharide in the ground substance of cancer stroma according to the pattern of growth of Brown-Pearce carcinoma of the rabbit, and empahsized that young connective tissues composed mainly of chondroitin sulfate were favourable to the growth of tumor cells. It is highly interesting that in the course of cancer growth, the connective tissue of cancer stroma changes its character according to the age of the host. These facts are of special interest in relation to many clinical and statistical findings that the growth tempo of the cancer tissue is influenced by the age of the host[23)34)37)]. The fact that autopsy cases in which cancer tissue of smaller area was found incidentally were of older age suggests that the cancer tissue may grow more slowly in older age.

Some authors suggested that the growth of cancer tissue may be inhibited by vigorous proliferation of the interstitium[9)10)11)13)14)]. However, other authors considered that vigorously proliferating stromal connective tissue was favorable to the growth of tumor cells[27)36)49)], namely, the slower tempo of cancer growth in older

age is considered to be due to the chemical character of the interstitium above mentioned beside the character of cancer cells themselves.

Tauchi, one of the authors, presented the concept of "inhibitory factor for cell division" by the investigation of senile atrophy of organs and tissues[44)45)46)], and discussed that this conception of the inhibitory factors was generally applicable more to ordinary tissue cells than to cancer cells.

From this point of view, the difference in tempo of cancer growth according to age may be considered to be influenced rather by the character of the interstitium, and this difference may lead to various pictures of cancer histology according to age. In this case, we have to remember the findings of Matsuyama[18)], who found the interesting close correlation between the morphological character of epithelial cells of the bronchi and that of subepithelial connective tissue.

From the above, the authors concluded that the histological characteristics of cancer tissue seen in older age was mainly influenced by the character of interstitia which become characterized in the course of cancer growth in older age.

3) **On the influence of age upon the appearance of small cancer cells.**

Tauchi et al.[42)43)] and Sato[31)] reported that the cancer cells became smaller in size in the course of cancer growth, by the comparative examination of surgical and autopsy cases. The authors reexamined larger number of cases of gastric cancer and reconfirmed that the picture of non-typical adenocarcinoma came to be found more frequently in the course of cancer growth and that the picture of small cancer cells was more often seen in older age. It was also noticed that there was no close correlation between degree of appearance of the picture of non-typical adenocarcinoma composed of small cancer cells and character of its interstitium. On the contrary, in case of carcinoma disseminata, degree of appearance of small cancer cells depended rather on the sclerotic grade of the interstitium than on the age of the host.

Tauchi et al[43)]. and Sato[31)] considered previously that the picture of carcinoma disseminata was more reversible and that of non-typical adenocarcinoma more irreversible. Tauchi[42)] considered that small cancer cells might be irreversible.

From some age differences in above mentioned relationships between the character of cancer cells and interstitium, the cancer cells were considered to become small in size from the character of interstitium in case of carcinoma disseminata, and from general conditions in case of non-typical adenocarcinoma. This consideration may be reasonable because of the fact that the cancer cells are scatteringly seen in the former case and gathered in the latter. Since the small cancer cells are considered to be reversible on one hand, and to be irreversible on the other, it is obvious that there are many problems to be clarified on this subjects of small cancer cells. No hasty conclusion can be drawn about nature of the small cancer cells.

Conclusion

The histological picture of the gastric cancer tissue was simple in earlier stage of the disease but became varied in later stage. This is considered to be due to appearance of various kinds of picture being derived from the original cancer histology in the course of its growth. The pattern of the appearance of those pictures is considered to be influenced by the enviromental condition of cancer growth, and some interesting findings were obtained in the pattern of appearance of some cancer histology according to age.

In autopsy cases, it was considered that the most characteristic picture in old age was typical adenocarcinoma with fine fibrillar interstitium and in younger cases carcinoma disseminata or mucous producing cancer with fibrous stroma. In autopsy case of younger age, there was generally seen marked proliferative picture of the stroma accompanied with even other histological pictures of cancer.

The histological pictures were considered to show that the cancer cells grew slowly with a picture of more complete differentiation of the cells in old age and rapidly with a picture of less complete differentiation in younger age. It may be considered that variation of the character of cancer cells according to age was due to the different changing process of the interstitium which is influenced by age factor in the course of cancer growth. It was found that some of cancer cells became smaller in size in the course of cancer growth. In case of carcinoma disseminata, small cancer cells appeared in relation to the character of the interstitium but not to age factor. However, in case of non-typical adenocarcinoma, small cancer cells appeared more frequently in autopsy cases of older age than of younger age, being less influenced by the character of the interstitium.

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Explanation of Plate XXI

Fig. 1 Mucous producing cancer with fibrous interstitium (Sek. C. 964, 36 year old female).
Fig. 2 Typical adenocarcinoma with fibrillar interstitium (Sek. 3339, 64 year old male).
Fig. 3 Non-typical adenocarcinoma (Sek. 3225, 45 year old female).
Fig. 4 Non-typical adenocarcinoma consisting of smaller cancer cells (Sek. C. 764, 64 year old male). All figures are of sections stained with H-E.

Fig. 1

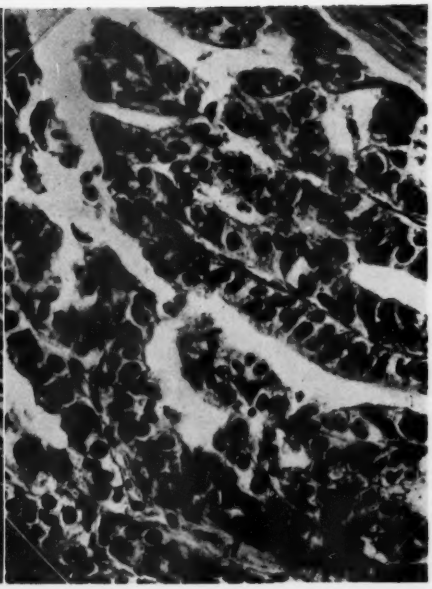

Fig. 2

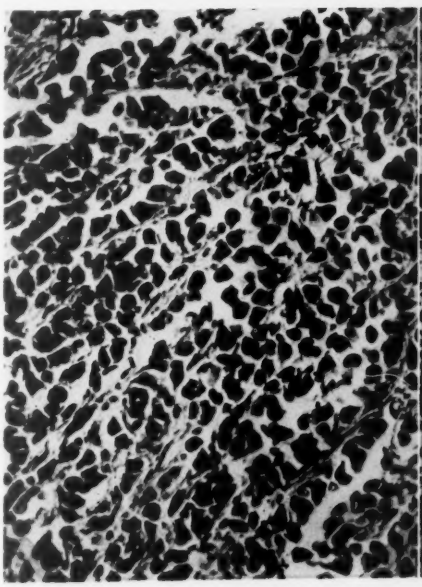

Fig. 3

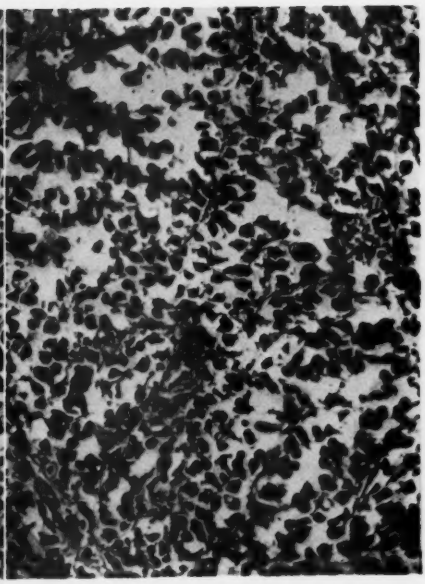

Fig. 4

[GANN, 51, 285~300; September, 1960]

STUDIES ON THE MODE OF ACTION OF RC4, A NEW ANTICANCER AGENT CONTAINING ETHYLENIMINO RING. II. ON THE INVERSE RELATIONSHIP BETWEEN INHIBITORY ACTIVITY ON ANAEROBIC GLYCOLYSIS AND ANTICANCER EFFECT OF RC4 ON EHRLICH ASCITES TUMOR CELLS

MOTOHIRO MARUYAMA
(Takamine Laboratory, Sankyo Co., Ltd., Tokyo)

It is already known that some of the ethyleneimines are highly active in glycolysis inhibition, and others are much less active.[1)2)]

In the previous report[3)] Maruyama and Uchida examined the characteristics of RC4 and related compounds, and recognized a similar fact. That is to say, they observed a strong glycolysis inhibiting activity in o-RC4, but hardly in RC4 or in TEPA. At the same time, however, it was found that the activities of latter solutions were gradually intensified, and eventually reached to that of o-RC4 solution, by preserving their aqueous solutions at a low temperature, for 2 weeks or more. The same tendency was noticed not only in the glycolysis inhibiting activity but also in action on some other enzymes. Hence they presumed that the difference in the glycolysis inhibiting characteristic of ethyleneimines at the time of dissolution was nothing but a disparity in the velocity of appearance of such characteristic.[3)] This presumption was supported by confirmation of the fact, which shall be reported in subsequent communications[4)] that the glycolysis inhibiting activities of RC4 and related compounds are intensified along with cleavage of ethyleneimino rings.

Holzer and his associates[1,5-7)] suppose that the anticancer effect of E39 may be derived from its glycolsis inhibiting activity. If this supposition is true, the same will apply to the case of RC4 from the above mentioned reason; thus, RC4 may presumably start its anticancer action after being transformed *in vivo* to a substance like that which appeared in preserved solution. Then, in order to clarify this possibility, the anticancer effects of the fresh and preserved solutions of RC4 were compared *in vitro* as well as *in vivo*. Unexpectedly, the anticancer effect of the

The following abbreviations are used in the present paper: RC4=O,O′-p-phenylene-N,N′,N″,N‴-tetraethylenetetraimido-diphosphate: o-RC4=O,O′-o-phenylene-N-ethyleneimidophosphate; TEPA=N, N′, N″-triethylenephosphoramide; Thio-TEPA=N, N′, N″-triethylenethiophosphoramide; NMO=nitrogenmustard-N-oxide; E39=2, 5-bisethyleneimino-3, 6-propioxy-1, 4-benzoquinone; TEM=2, 4, 6-triethyleneimino-1, 3, 5-triazine; ATP=adenosinetriphosphate; DPN=diphosphopyridime nucleotide; HDP=hexosediphosphate; TCA=trichloracetic acid

fresh solution was far stronger than that of the preserved solution in all cases, and it was concluded that the anticancer activity of RC4 was not related to its glycolysis inhibiting activity. In addition, as described in the present report, the glycolysis inhibiting activity of RC4 was antagonized by addition of either nicotinamide or DPN, which characteristic closely resembles those of E39[5] or TEM[8].

Materials

RC4, prepared by Dr. Genshun Sunagawa in our laboratory, was stored at 10°C in vacuo, and its aqueous solution was preserved at 2°C. NMO was a product of the Yoshitomi Pharmaceutical Company. It was neutralized with diluted NaOH immediately before use. DPN (90% pure) was obtained from the General Biochemicals, Inc. ATP (dipotassium salt) and HDP (di Barium salt) were purchased from the Nutritional Biochemicals Corp. The Barium of HDP was precipitated with H_2SO_4 and the free acid thus obtained and the aqueous solution of ATP were neutralized with $KHCO_3$ aqueous solution before use. The Ehrlich ascites tumor cells were transplanted into dd-mice, and harvested on 7 to 9 days after inoculation.

Methods and Results

Variation in the Inhibitory Activity of RC4 Aqueous Solution When Preserved at 37.5°C against Anaerobic Glycolysis.

After approximately two weeks of preservation at 2°C, 10% aqueous solution of RC4 acquires an activity about 50 times as strong as its initial activity, and after that, an approximately equal activity is maintained for two weeks.[3] The preserved solution in this period will hereinafter be referred to as simply "stock solution." In order to ascertain whether RC4 *in vivo* underwent a similar transformation and then exerted an anticancer action, it was first examined if the variation in the activity which had been observed in low temperature (2°C) preservation was also observed under near body heat. Ten percent and 0.5% aqueous solutions of RC4 were preserved at 37.5°C continuously shaking, and were sampled at certain intervals, the glycolysis inhibiting activity being measured at each time. Measurement of anaerobic glycolysis was conducted by an conventional manometric method. The preserved solution of RC4 was diluted to various concentrations, and each diluted solution was put into the main flask together with tumor cells. After temperature equilibration, the glucose in the side arm was tipped into the main flask and the CO_2 evolution was measured. By comparing this quantity with that of CO_2 output in a flask in which RC4 was not contained, the degree of inhibition (I) was computed, and the 50% inhibitory concentration (I_{50}) was obtained in accordance with the conventional method given in the preceding report.[3] The 50% inhibitory concentrations before and after preservation being represented by I^t_{50} and I^0_{50}, respectively,

the ratio between them (I^t_{50}/I^0_{50}) was designed as 'increasing degree of inhibition'. When the 0.5% solution was preserved, the initial inhibitory activity was so weak that a considerable quantity of the sample solution had to be added. In such a case, a half amount of Krebs-Ringer solution of double concentration was used, and the excess amount of the sample was compensated for with the curtailed quantity of the buffer solution.

Fig. 1 shows the results of the above described experiment. The unit on the horizontal axis is "hour".

The 10% aqueous solution achieved its maximum activity after approximately 8 hours of preservation, and after that it gradually lost its activity. In the case of the 0.5% aqueous solution, the variation in the activity was so slow that it took 30 hours to reach the peak, while an almost constantly high activity continued for more than 10 hours after that. The dotted line represents the variation in the activity of the 10% aqueous solution preserved at 2°C; in this case the unit on the horizontal is "day".

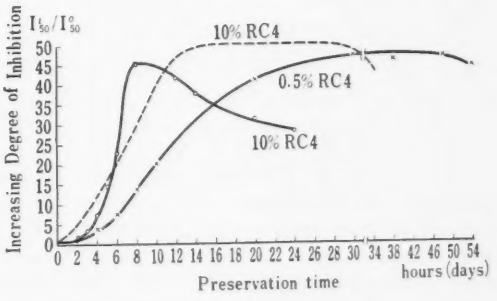

Fig. 1. Effect of preserving of RC4 aqueous solution before test at 37.5°C. or 2°C., on its inhibitory activity against anaerobic glycolysis of Ehrlich ascites tumor cells. 'Increasing degree of inhibition' is presented as I^t_{50}/I^0_{50}, where I^0_{50} and I^t_{50} show 50% inhibitory dose of obsewed and initial time of preservation, respectively. Solid or dotted line indicates the result of preservation at 37.5°C (time unit: hour) or 2°C (time unit: day), respectively.

The maximum activity achieved did not vary conspicuously with the temperature or the concentration during preservation. That is to say, if the problem of excretion is set aside for the present, it is presumable that the same variation as in the case of low temperature preservation can take place *in vivo*.

Effect of Nicotinamide on the Inhibition of Anaerobic Glycolysis by RC4.

It is reported by some workers that TEM[8] or E39[5-7] lower the DPN level of tumor cells and their inhibitory action against anaerobic glycolysis is antagonized by addition of nicotinamide. The mechanism of glycolysis inhibition by RC4 has been examined from this aspect.

Fig. 2 and Fig. 3 show the glycolytic activity of tumor cells incubated with various concentrations of RC4 fresh or stock solution. RC4 put into the side arm together with glucose, and was tipped into the main flask after temperature equilibration. It is a characteristic feature that the degree of inhibition increases along with the progress of the reaction. The solid lines are the results in Krebs-Ringer-bicarbonate

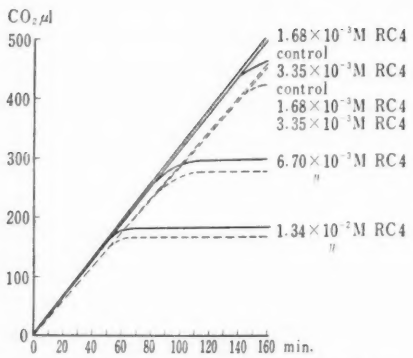

Fig. 2. Effect of RC4 fresh solution on anaerobic glycolysis of ascites tumor cells in Krebs-Ringer-bicarbonate solution (solid line), or in Hanks' solution fortified with ascites fluids (dotted line). Main flask: 1.5×10^7 cells in 2.5 ml of Krebs-Ringer-bicarbonate solution, or 1.2×10^7 cells in 1.0 ml. of ascites fluids, 0.2ml. of 4% $NaHCO_3$, and 1.3ml. of Hanks' solution. Side arm: 1.5% glucose 0.2 ml., RC4 aqueous solution or water 0.3 ml.

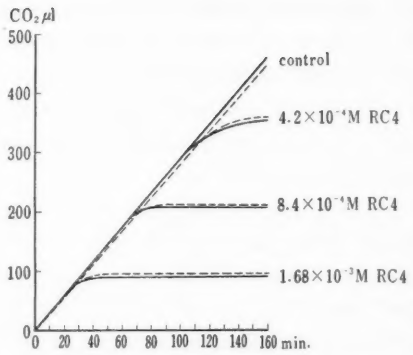

Fig. 3. Effect or RC4 stock solution on anaerobic glycolysis of ascites tumor cells in Krebs-Ringer-bicarbsnate solution (solid line), or in Hanks' solution fortified with ascites fluids (dotted line). Main flask: 1.5×10^7 cells in 2.5ml. of Krebs-Ringer-bicarbonate solution, or 1.2×10^7 cells in 1.0 ml. of ascites, fluids, 0.2ml. of 4% $NaHCO_3$, and 1.3 ml of Hanks' solution, side arm: 1.5% glucose 0.2ml., RC4 aqueous solution or water 0.3 ml.

solution for the medium, and the dotted lines are those in Hanks' solution fortified with ascites fluid. It is recognized that the activities of RC4 in the former medium are not significantly different from those of RC4 in the latter medium. Fig. 4 shows the effect of nicotinamide. Nicotinamide, RC4 stock solution and glucose were put into the side arm, and were tipped into the main flask after temperature equilibration.

Nicotinamide of 1~25 mM* neither inhibited nor activated the glycolysis of tumor cells. On the other hand, 1 mM RC4 completely inhibited the glycolysis about 40 minutes after addition. In the case of 1 mM RC4 and 1~25 mM nicotinamide being applied simultaneously, the presence of 1 mM nicotinamide little affected the inhibitory activity of RC4, but 5~25 mM nicotinamide could almost completely reverse the inhibitory action of RC4. Namely, nicotinamide antagonizes the inhibition of glycolysis by RC4. Furthermore, it was observed that nicotinamide also reversed the inhibition of glycolysis of ascites tumor cells by NMO (Fig. 5).

The Inhibitory Action of RC4 against the Glycolysis of Tumor Cell Homogenates and Effect of Nicotinamide and DPN

Since the mode of glycolysis inhibition by RC4 on cell level was made clear to

* For the sake of distinction, M, mM and μM are used as units of concentration, and mole, m mole and μ mole as those of quantity.

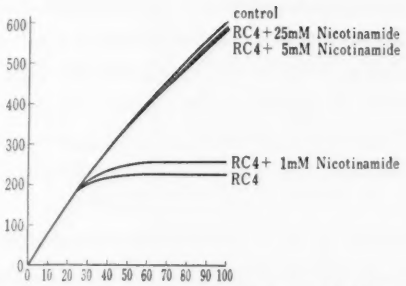

Fig. 4. Effect of nicotinamide on inhibition of anaerobic glycolysis of ascites tumor cells by 1 mM RC4 stock solution. Main flask: 2.5×10^7 cells in 2.3ml. of Krebs-Ringer-bicarbonate solution. Side arm: 1.5% glucose 0.2ml., nicotinamide and/or RC4 and/or water 0.5ml.

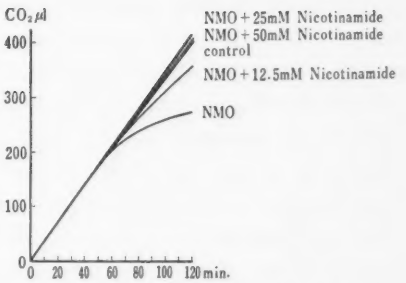

Fig. 5. Effect of nicotinamide on inhibition of anaerobic glycolysis of ascites tumor cells by 0.75mM NMO. Main flask: 2.0×10^7 cells in 2.3ml. of Krbs-Ringer-bicarbonaie solution. Side arm: 1.5% glucose 0.2ml., nicotinamide ann/or NMO and/or water 0.5ml.

some extent, similar examination was attempted on the homogenate level. The tumor cells were separated from blood corpuscles by washing with ice cold 0.9% KCl (adjusted to pH 7.4 with $KHCO_3$) 3 to 4 times, and the number of cells was counted. The cells were suspended in 0.9% KCl containing about $5\sim10\times10^7$ cells/ml, and after the suspension has been fully homogenized with a Potter-type homogenizer for about 10 minutes, it was diluted with 0.9% KCl to 1.5×10^7 cells/ml equivalent.

Measurement of anaerobic glycolysis of homogenates was conducted by use of the similar medium to Le Page[9] (Table 1). Since glucose reversed slightly the glycolysis inhibiting action of RC4, 16.6 μ moles of glucose was applied as in the experiment on the cell level. Furthermore, protecting efficiency of the cofactors in the medium against RC4 inhibition was examined by means of reducing or completely removing

Table 1. Composition of the anaerobic glycolysis standard medium

Main flask	Final molarity
Potassium phophate	0.0024
Potassium bicarbonate	0.025
Nicotinamide	0.040
Adenosinetriphosphate*	0.00033
Diphosphopyridinenucleotide	0.00020
Hexosediphosphate*	0.0020
Potassium pyruvate	0.0075
Magnesium chloride	0.0066
Potassium fluoride	0.010
Homogenate in 0.9% Kcl	
Side Arm: Glucose	0.0055

Water to make a fimal volume of 3.0 ml. Gas mixture: 95% N_2; 5% CO_2 * Potassium salt

them from the medium. After 10 minutes equilibration at 37.5°C, the reaction was allowed to proceed for 90 minutes and the CO_2 evolution was measured at 10 minutes intervals. At the begining and end of the reaction, TCA in the side arm was tipped into the main flask, and using the supernatants of deproteinized reaction mixtures, lactate was estimated by the method of Barker-Summerson[10] and inorganic phosphorus, by the Takahashi method[12] which was a modification of the method of Martin-Doty.[11]

Thus the quantity of lactate formed and that of inorganic phosphorus esterified were computed. Since gas evolution not due to glycolysis was negligible, it was ignored. For colorimetry, the Hitach EPU II spectrophotometer was employed.

Table 2 gives the results of experiments on the complete system. Fresh and stock solutions of RC4 were so weak in the activity that they slightly inhibited formation of lactate and so CO_2 at such high concentration as 2.4×10^{-2} M and 1.2×10^{-2} M respectively, but they stimulated somewhat the uptake of inorganic phosphorus. While a similar tendency was observed in *o*-RC4, NMO inhibited the uptake of

Table 2. Effect of RC4 or related agents on araerobic glycolysis of Ehrlich ascites tumor cell homogenates. (In Complete System)

Exp. No.	Final Conc. mM	CO_2 evolution μ moles	Lactate produced μ moles	Net P esterified μ moles
1	0	12.6	11.6	6.5
	RC4 fresh solution			
	24	11.8	10.9	7.8
	2.4	12.9	11.7	6.5
	0.24	13.2	12.0	6.4
	RC4 stock solution			
	12	12.1	11.1	9.5
	2.4	14.5	13.3	7.4
	0.24	14.5	13.4	7.1
2	0	10.6	10.0	7.7
	〃	10.3	10.0	7.5
	NMO fresh solution			
	0.75	4.4	4.2	0.3
	〃	4.7	4.4	0
	0.38	4.6	4.5	0.8
	〃	4.8	4.7	0.8
	0.19	6.6	6.4	3.9
	〃	6.7	6.5	3.9
3	0	6.2		5.4
	o-RC4 fresh solution			
	50	2.4		10.9
	5	8.0		8.1
	0.5	6.8		8.2

Table 3. Effect of decreasing of cofactor lebels on inhibition of anaerobic glycelysis of Ehrlich ascites tumor cell homogenates by RC4 stock solution.

System		CO_2 evolution μ moles	Lactate produced μ moles
Complete system-KF,-HDP 1/10 ATP and pyruvate		14.5	13.3
〃	+RC 4 12 mM	12.0	11.8
〃	+ 〃 6 〃	14.6	13.5
〃	+ 〃 3 〃	14.4	13.0
		CO_2 evolution μ moles	Inhibition %
Complete system-KF		27.8	
〃	+RC 4 1mM	28.0	−0.7
Complete system-KF, 1/10 DPN		27.5	
〃	+RC 4 1mM	26.5	3.7
Complete system-KF-Nicotinamide		27.2	
〃	+RC 4 1mM	23.6	14.1
Complete system-RF-Nicotinamide, 1/10 DPN		15.3	
〃	+KC 4 1mM	11.1	27.5
Anaerobic glycolysis by cells		29.4	
〃	+RC 4 1mM	10.0	46.0

Homogenate: equivalint to 3.0×10^7 cells. Reaction time: 0~90min.

inorganic phosphorus even more than it inhibited the formation of lactate or CO_2. NMO at the concentration of 2×10^{-4}M inhibited phosphorylation approximately 50%. Despite the glycolysis of the tumor cells was completely inhibited by 1 mM of RC4 stock solution 40 minutes after the start of the reaction, even 12 mM of RC4 stock solution did not conspicuously inhibit the glycolysis of the tumor homogenates. The probable reason is that, since several sorts of cofactors are contained at high concentration in LePage's medium, especially nicotinamide being contained in a large quantity, the inhibitory action is antagonized by such cofactors. The results of examination on this point are given in Table 3.

The quantity of the homogenates used was equivalent to 3×10^7 cells and the reaction was continued for 90 minutes. Since all of the following experiments were conducted in the incomplete systems, phosphorylation was not measured. As the presence of KF considerably lowers the activity, KF was added in no case.

In a system which omitted HDP as well as KF from the complete system and lowered the ATP and pyruvate levels to 1/10, the inhibitory activity of RC4 was very little as in the case of the complete system. Reduction of the DPN level by 1/10 neither remarkably increased the inhibitory activity of RC4. However, by removing nicotinamide, the inhibition by 1 mM stock solution was raised to 14%,

and in a system which was lacking nicotinamide and the DPN level was reduced by 1/10, RC4 stock solution displayed 27.5% inhibition at 1 mM. On the other hand, glycolysis of cells is inhibited by the same stock solution of the same concentration 46% (0–90 minutes). Hence the principal reason why the inhibition by RC4 was not observed in the complete system is presumably due to the protective action of nicotinamide and DPN.

From the results of the experiments using the tumor cells and their homogenates, it was found that the mode of glycolysis inhibition by RC4 closely resembled the action of TEM and E39. Then it was studied whether such a glycolysis inhibitory activity was the source of the anticancer action of RC4, by comparing the anticancer activities of stock and fresh solutions.

In addition, NMO inhibited anaerobic glycolysis of ascites tumor cell homogenates strongly, and, despite nicotinamide could protect glycolysis of the tumor cells from the inhibitory action of NMO, it could not reverse the inhibitory action of NMO on glycolysis of the tumor cell homogenates, although its concentration in the medium was elevated to 100 mM (Fig. 6). Accordingly, the mechanisms of action of NMO on anaerobic glycolysis seems to be different from those of ethyleneimines.

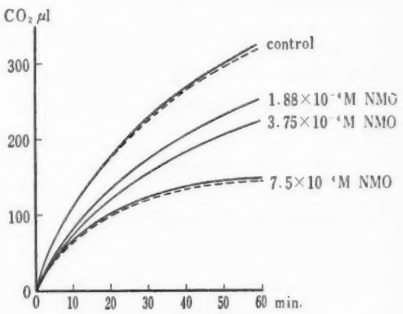

Fig. 6. Effect of NMO on anaerobic glycolysis of ascites tumor cell homogenates instandard medium (solid line; 40 mM nicotinamide) or modified medium (dotted line; nicotinamide concentration was elevated to 100 mM, other composition was the same as standard medium). Homogenates equivaletent to 2.5×10^7 cells were added to each flask.

The Anticancer Activities of Fresh and Stock Solutions of RC4 in In Vitro Test

By improving Fukuoka and her collaborator's method,[13,14] tests were conducted under a condition closer to the physiological condition. Ascites tumor cells, 7 days after transplantation on dd-mice, were gathered in a syringe in which a minute amount of heparin had been added, and the cells were centrifugally separated from the ascites fluid. After removing most of the blood-corpuscles by washing the cells with Krebs-Ringer solution 2 or 3 times, the cells were resuspended in the ascites fluid, which had been separated, to make the final cell concentration of 1×10^8 cells/ml. (A).

Both fresh and stock solutions of RC4 were diluted with Hanks' solution (B). The composition of Hanks' solution is given in Table 4.

A and B, 1 ml each, were mixed with each other and, after being incubated for 1 or 2 hours at 37.5°C, the mixture was diluted with 6 ml of ice cold Hanks' solution.

Table 4. Compositon of Hanks' solution

NaCl	8.0 g
KCl	0.4 g
$MgSO_4 \cdot 7H_2O$	2.0 g
$CaCl_2$	1.4 g
Na_2HPO_4	0.06 g
KH_2PO_4	0.06 g
$NaHCO_3$	0.35 g
Glucose	1.0 g
Dihydsostreptmycin sulfate	0.04 g
Penicillin-G-Ksalt	1,000,000 units
0.1 % phenol red	20 ml

Redistilled water to make a final volume of 1000ml.

Then 0.2 ml of the diluted mixture was transplanted intaraperitoneally into a dd-mouse, and the anticancer activity was determined by body weight courve and survival period. The stock solution used had been preserved for 13 days (for 1 hour incubation) or 14 days (for 2 hours incubation). Both fresh and stock solutions were diluted to 7 different degrees of concentration, and 10 mice were used for each concentration of each type—fresh or stock—of solution. Into the control mice, the cells incubated in Hanks' solution which did not contain RC4, were transplanted.

Fig. 7 and Fig. 8 represent the results of the above mentioned test. Contrary to the expectation, the anticancer effect of the fresh solution was stronger, irrespective of the incubation time. As already stated, the inhibitory activity of RC4 on anaerobic glycolysis did not change, if Krebs-Ringer-bicarbonate solution was substituted for Hanks' solution fortified with ascites fluid, as the medium. (Fig. 2 and Fig. 3)

In the case of one hour's incubation, although the fresh solution did not inhibit

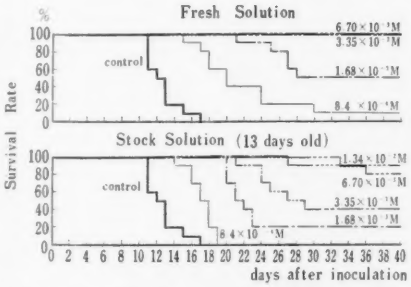

Fig. 7. Comparison of antitumor effect between fresh and stock solutions of RC4 against Ehrlich ascites tumor cells (in vitro test; incubation time: 1 hr) Figures are final concentrations at the time of incubation.

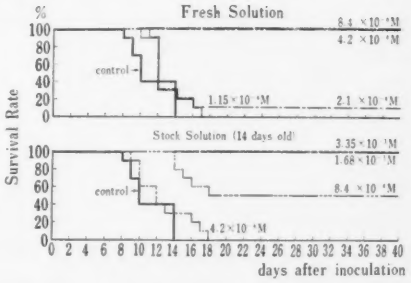

Fig. 8. Comparison of antitumor effect between fresh and stock solutions of RC4 against Ehrlich ascites tumor cells (in vitro test; incubation time; 2 hrs). Figures are final concentrations at the time of incubation.

glycolysis at 6.70×10^{-3}M or lower concentrations (Fig. 2), it could inhibit the growth of the transplanted tumor cells completely at 3.35×10^{-3} M (Fig. 7). On the other hand, the stock solution could inhibit glycolysis completely at 1.68×10^{-3} M (Fig. 3), but it reterded the growth of the transplanted tumor cells only to a slight degree at the same concentration (Fig. 8). Similar characteristics were found in the case of two hours' incubation.

Although a minute quantity of RC4 was injected with the inoculation of cells, it was concluded that anticancer activity of this amount of RC4 should be negligible. For, injection of RC4 twice as much as the maximum of the above mentioned quantity with the control cells into mouse gave no anticancer effect. Preservation of RC4 aqueous solution for 2 hours at the temperature of 37.5°C caused only negligible variation in the activity of the solution (Fig. 1). From these findings it can well be assumed that the results represented in Fig. 7 and Fig. 8 accurately reflect the *in vitro* effects of both fresh and stock solutions. In addition, even if the incubation was conducted under anaerobic condition, the similar results were obtained. Hence it can be reasonably be concluded that the antiglycolytic characteristic of RC4 is not the main feature of its anticancer action *in vitro*.

Reliability of the In Vitro Test.

Examination has been made on a few points which should be taken into consideration in studying to what extent the results of the *in vitro* test would help understand the action of the RC4 solution *in vivo*.

Influence of dilution: After contact with the agents, the cell suspension was diluted. The first problem was whether the glycolytic activity which had been inhibited would be recovered or not by the dilution. If the inhibition by RC4 is reversible, the inhibited activity can possibly be recovered by dilution. On the other hand, since it is a common practice in *in vivo* tests to apply agents for several days, the agents will have more opportunities to be in contact with cells and the hours of contact will be longer than in *in vitro* test, hence a reversible inhibition can exercise a considerable influence, which may become the cause of disparity between the results of *in vivo* and *in vitro* tests.

Fig. 9 represents the results of examination on this point. Washed cells were resuspended in ascites fluid at the rate of 1.0×10^{8} cells/ml (A), and RC4 fresh or stock solution was diluted with Hanks' solution (B).

Mixtures were made each of 1.0 ml of A and B, to render the final concentrations of RC4 1.34×10^{-2}M, 6.70×10^{-3} M and 3.35×10^{-3} M for the fresh solution and 1.68×10^{-3}M, 8.4×10^{-4}M and 4.2×10^{-4}M for the stock solution, respectively. As the control, Hanks' solution which did not cantain RC4 was used as B. After replacing the gas phase with 95% N_2–5% CO_2 (it took 10 minutes for gas exchange), incubation was allowed to proceed for 60 minutes at 37.5°C, continuously shaking. This

experimental condition was the same as that of *in vitro* test.

Immediately after the end of the incubation, 6 ml of ice cold Hanks' solution was poured into incubation tube, and thus each mixture was diluted to 4 times. Two ml of this diluted mixture was put into main compartment of Warburg flask which already contained 0.6 ml of Hanks' solution and 0.2 ml of 4% $NaHCO_3$ in the main compartment and 0.2 ml of 1.5% glucose in side arm. The anaerobic glycolytic activity of the incubated cells was determined by a conventional method. It took 25 minutes to start measuring of the activity from the end of the incubation.

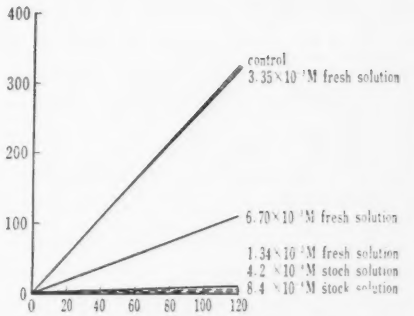

Fig. 9. Anaerobic glycolytic activity of ascites tumor cells diluted to four times after incubating with various concentrations of RC4 fresh or stock solution for 60 minutes at the same condition of in vitro test. Figures show final concentration of RC4 before dilution. For measurement of the activity, 2.5×10^7 diluted ascites cells were added to each warburg's flask.

The cells incubated with either the stock solutions or the 1.34×10^{-2} M fresh solution had lost their glycolytic activities, which facts tell that the inhibited glycolytic activity not recovered by dilution. Furthermore, the activities of the cells incubated with 3.35×10^{-3}M fresh solution did not differ from that of control cells, and the cells incubated with the 6.70×10^{-3}M fresh solution had about 33% activity of that of control cells. These results show that the inhibition of the activity of RC4 is neither recovered nor strengthened after the dilution.

Influence of cell lysis: Any alkalyting agent has more or less hemolytic activity. Therefore, it was examined whether such an unspecific action would influence the results of *in vitro* tests. After washing the citrated blood withdrawn from the vena antibrachii of a dog three times with Krebs-Ringer solution containing citric acid, the erythrocytes were suspended in Krebs-Ringer solution to make the quantity of the suspension equal to that of the blood before washing. Fresh and stock solutions of RC4, and such highly hemolytic surface active agents as Rapizol-B-80 (anionic)*, Hyamine (cathionic)**, and Triton-X-100 (nonionic)***, each dissolved in Krebs-Ringer solution, were used as test solution.

To 5 ml of each test solution, which was successively twice diluted with Krebs-Ringer solution was added 0.1 ml of the erythrocyte suspension, and the mixture was let stand for 1 hour at 37.5°C. Then the color intensity of the supernatant, obtained by centrifugation for 10 minutes at 2000 g, was measured at 541 mμ, thus the minimum hemolytic concentration of each test solution being obtained. At the same time, the anticancer effect of each of the above mentioned agents in *in vitro* tests was exa-

Table 5. Effect of cell-lytic action of RC4 on the estimation of its 'in vitro' antitumor activity.

Agents	Minimum haemolytical concentration(=M.H.C.) %	-log T of haemolyzate at M.H.C. $\lambda_{541\ m\mu}$	Anti-tumor effect		
			Final conc. %	Evaluation*	Toxicity
RC4 fresh solution	2.5	0.061	0.0156 (M.H.C./160)	##	—
Rapizol-B-80 (anionic)	0.00078	0.915	0.0001 (M.H.C./8)	—	—
Hyamine (cathionic)	0.00078	0.155	0.0001 (M.H.C./8)	—	—
Triton-x-100 (nonionic)	0.00625	0.285	0.00078 (M.H.C./8)	—	—

* ##: Complete inhibition of tumor growth; —: No effect.

mined, and these results are summarized in Table 5. RC4 has a slight hemolytic activity at 2.5% or more, on the other hand, same of 0.0156% (4.2×10^{-4} M), which is 1/160 of the former figure, displayed a complete anticancer effect. However, the three other surface active agents, even at 1/8 of minimum hemolytical concentration, exercised no anticancer action at all. When the surface active agents 4 times as much as the quantity which would be injected to a mouse together with the tumor cells was injected intraperitoneally into a healthy mouse without tumor cells, toxic death did not occur. Hence it is presumed that the cell lysis is not related to the results of the *in vitro* tests of RC4.

These results indicate that the actions of RC4 fresh or stock solutions *in vivo* can be inferred from their actions in *in vitro* test with considerable accuracy, and suggest that the fresh solution may probably be more active also *in vivo*.

Comparison of Effects of RC4 Fresh and Stock Solutions by In Vivo Test

What is worthy of note regarding the *in vivo* test is the variation in the activity of RC4 injected into a living body. Probably the fresh solution *in vivo* gradually acquires the characteristics of the stock solution, and the stock solution by and by loses its activity, then, the velocity of the variation in the activity should be taken into consideration in interpreting the results obtained by *in vivo* test. That is to say, if the variation is rapid, what has been considered the action of the fresh solution may have originated after the solution has been converted to a stock-solution-like substance.

Koyama and his associates[15] measured the blood level of RC4 after intravenous injection of P^{32}-RC4 for human beings by following its radioactivity, and it was confirmed that the radioactivity decreased by 1/10 or less in several hours after the administration. Since the phoshorus in RC4 is hardly isolated in aqueous solution[4], the rise and fall of RC4 *in vivo* may be inferred to a some extent from such a result.

* Sodium dialkylsufosuccinate ** Benzethonium chloride ***Polyoxyethylenenonylphenol

On the one hand, the results represented in Fig. 1 tell that the variation in the activity becomes slower as the concentration is lowered: when preserved at 37.5°C, 0.5% RC4 takes more than 30 hours to achieve its maximum activity, which lasts for more than 10 hours after being achieved. On the other hand, in the *in vivo* test, maximm fluid level is presumed after administration of RC4 even at 100 mg/kg about 0.2% or less, when total fluid quantity is assumed as 1/20 of body weight. Afterwards the adminisuration the fluid level may rapidly become lower, probably down to 1/10 or less of the initial level several hours later, as was stated above. Therefore, even if the variation in the activity *in vivo* is ignored in interpreting the experiment results, no great error will follow. To make clear this point to some extent, following experiment was condcuted and its results are presented in Table 6.

RC4 fresh or stock solution at the doses of 100, 150 and 200 mg/kg respectively, was injected intraperitoneally into dd-mice, which had been inoculated with Ehrlich ascites tumor cells seven days before, with average body weight of 30.0±1.0 g. Four hours after the injection, the ascites cells were separated and washed. The anaerobic glycolysis and endogenous respiration of the washed cells of each mouse was measured by the conventional method[3]. Both activities of the cells which were obtained from the mouse administered with the fresh solution were of the same

Table 6. Anaerobic glycolysis and endgenous respiration of ascites tumor cells obtained from the mice, which had been intraperitoneally injected various amounts of RC4 fresh or stock solution before four hours

	Amount of RC4 injected	Number of Animal	Anaerbic glycolysis $CO_2\mu l/hr/2.5\times10^7$ cells	Endogenous respiration $O_2\mu l/hr/5.0\times10^7$ cells
Fresh solution	control	1	226	135
		2	210	125
	100m g/kg	3	220	120
		4	207	127
	150 mg/kg	5	240	140
		6	228	132
	200 mg/kg	7	210	128
		8	235	142
Stock solution	control	9	288	144
		10	267	132
	100 mg/kg	11	250	139
		12	272	140
	150 mg/kg	13	201	144
		14	191	142
	200 mg/kg	15	148	155
		16	123	128

degree as those of control cells. On the contrary, the glycoltic activity of the cells, which were obtained from the mouse administered with 150 or 200 mg/kg of the stock solution, was apprently reduced as compared with that of cotrol cells, but no significant difference was found in the respiratory activities among these three groups of the cells. From these results, it may be concluded that the stock solution inhibits anaerobic glycolysis of the ascites tumor cells *in vivo* more strongly than the fresh solution does.

The test was conducted in accordance with an ordinary method. Two million cells of the ascites tumor were inoculated into each dd-mouse intraperitoneally, weighing about 20 g, 8 weeks old, ten mice in a group, and from 48 hours later on, either fresh or stock solution was injected into the peritoneal cavity of each mouse once a day for 6 successive days. The anticancer activities were estimated by surival curves or body weight curves for 2 months after that. At the time of inoculation of cells and injection of agents, penicillin and streptomycin were injected in order to avoid infection. Fig. 10 represents the results of this test. Administration of 17.8–90 mg/kg of the stock solution gave no anticancer effect, on the contrary 17.8 mg/kg of the fresh solution gave a 70% survival rate and 40 mg/kg or more of same decreased the rate of death due to cancer down to 10%. On the other hand, administration of 60 mg/kg or more caused death due to the secondary toxic effect of the agent.

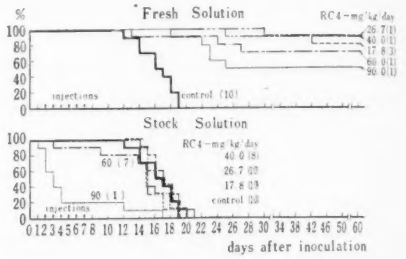

Fig. 10. Comparison of antitumor effect between fresh and stock solutions of RC4 against Ehrlich ascites tumor cell. (in vivo) Figures in parenthesis are numbers of death animals caused by tumor.

The results of the *in vivo* test also indicated that the fresh solution had a far more powerful anticancer activity than the stock solution.

Discussion

Roitt[8] found that 10^{-3}M TEM inhibited anaerobic glycolysis of various ascites tumor cells, and that such an inhibition occurred with the decline of the DPN level and the inhibited glycolysis was recovered by the addition of nicotinamide. Holzer *et al.*[1] observed that various ethyleneimines inhibited anerobic glycolysis at such concentration as would not inhibit the respiration of tumor cells, and thus assumed that the anticancer activity of them was due to the glycolysis inhibiting activity. Nevertheless, since the glycolysis inhibitory activity of thio-TEPA was known to be exceedingly weak, they attributed its cause to the fact that the ethyleneimino rings of Thio-TEPA were combined with five valency phosphorus.

After examination of the characteristics of RC4 and related compounds, the author and his associates have arrived at a hypothesis[3] that these compounds acquire their glycolysis inhibiting activity after being transformed to a certain common active form and the disparity in the velocity of such transformation resulted in the difference in their characteristics at the time of dissolution; the grounds for such a hypothesis were the findings that even the ethyleneimines which were not active in glycolysis inhibition at the time of dissolution, acquired a higher activity equal to TEM by preserving their aqueous solutions at a low temperature,[3] and that the glycolysis inhibiting activity revealed itself along with the cleavage of the ethyleneimino rings.[4] The finding that the glycolysis inhibiting activity of E39 or TEM is more vigorous at a lower pH[1,5] may be explained by the above mentioned hypothesis, because the cleavage of the ethyleneimino ring progresses more rapidly under acid condition.[4] Furthermore the results in the present report show that the mode of action of RC4 stock solution against the anaerobic glycolysis of tumor cells and of their homogenates is closely similar to that of TEM or E39, and support the above hypothesis.

As was previously stated, though the significance of the glycolysis inhibiting activity of ethyleneimines is highly appreciated, it has not yet been established. In an attempt to make a step toward the solution of this problem, the author utilized the difference in characteristics between the fresh and stock solutions of RC4, namely their anticancer activities *in vitro* as well as *in vivo* examined. It has been concluded that, at least as far as RC4 is concerned, there is no unitary relationship between its glycolysis inhibiting action and its anticancer effect. Since the characteristics of glycolysis inhibition seem to be common to ethyleneimines as already stated, it may be possible to generalize the above mentioned conclusion about RC4 in the cases of other ethyleneimines, such as E39, TEM, etc.

Furthermore, a similar conclusion may be reached as to the activity of RC4 which increases by preservation. Of the enzymes examined in the preceding report,[3] the actions on alkaline phosphatase and specific ChE are not related to the anticancer activity of RC4, and only the action on nonspecific ChE may possibly be related to its essential property. The latter probability, however, requires a more detailed study.

In addition, the glycolysis inhibiting activity of NMO on tumor homogenates is not reversed by nicotinamide, and NMO inhibits the uptake of phosphorus more than it inhibits the formation of lactic acid. Considering these facts, the characteristics of NMO are obviously different from those of RC4, and the significance of the inhibitory activity of the former should be discussed from a different standpoint.

Summary

1) The inhibitory action of RC4 stock solution on anaerobic glycolysis of Ehrlich ascites tumor cells or their homogenate is antagonized by addition of nicotinamide

or DPN. The inhibitory activity of the fresh solution is exceedingly weak.

2) After contacting the ascites tumor cells with RC4 fresh and stock solutions in Hanks' solution fortified with ascites fluid, the tumor cells were transplated to mice, and the anticancer activites of the fresh and stock solutions were compared by observring whether the tumor cells had grown or not in the mice. The fresh solution, even at concentration as will not at all inhibit glycolysis, completely inhibited the growth of the tumor cells, while the stock solution, at a concentration as will perfectly inhibit glycolysis, did not completely inhibit growth of the tumor cells.

3) Forty-eight hours after inoculation of the ascites tumor cells into mice, the fresh or stock solution of RC4 was injected into the peritoneal cavity of each mouse, and their anticancer activity were examined. Only the fresh solution displayed a conspicuous effect.

4) From the above mentioned findings, it has been conculuded that the anticancer activity of RC4 is not related to its glycolysis inhibiting activity. Furthermore, a few more facts were found to suggest that the anti-glycolytic characteristics of ethylenemines have some common features. Hence, the conclusion concerning RC4 may possibly be generalized in the cases of other ethyleneimines.

5) Neither stimulation of alkaline phosphatase nor inhibition of specific ChE is related to the anticancer effect of RC4.

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STUDIES ON THE MODE OF ACTION OF RC4, A NEW ANTI-CANCER AGENT CONTAINING ETHYLENIMINO RING. III. A SPECTROPHOTOMETRIC STUDY ON STRUCTURAL CHANGES OF RC4 IN AQUEOUS SOLUTION

MOTOHIRO MARUYAMA
(Takamine Laboratory, Sankyo Co., Ltd., Tokyo)

In earlier papers on the characteristics of RC4[1] and related agents, it was presumed that the ethyleneimines would acquire such properties as to inhibit glycolysis or to stimulate alkaline phosphatase, after being converted to a certain active type[1)2)]. In order to substantiate this presumption, the correlation between the structural changes of RC4 in aqueous solution and the increase of the glycolysis inhibiting activity was studied from the viewpoint of spectral changes in ultraviolet and infrared absorptions, and the results of this study will be given here.

MATERIALS

RC4, prepared by Dr. Genshun Sunagawa in our Laboratory, was stored at 10°C in vacuo.

The Ehrlich ascites tumor cells were transplanted into dd-mice (Hamamatsu Farms), and harvested on 7 to 9 days after transplantation.

METHODS AND RESULTS

Variation in the Inhibitory Activity of RC4 against Anaerobic Glycolysis in the Course of the Preservation.

In order to ascertain whether the structural changes of RC4 in aqueous solution resulted solely from hydrolysis, that is a first order reaction, or from a higher order reaction, such as polymerization, RC4 aqueous solutions of different concentrations were preserved at 2°C, and the variation in the inhibitory activity against anaerobic glycolysis was examined at certain intervals in the course of the preservation. Measurement of glycolysis was conducted manometrically[1)], and RC4 was put into the main flask together with the tumor cells. After 10 minutes temperature equilibration, glucose in the side arm was tipped into main flask, and the reaction was allowed to proceed for 60 minutes at 37.5°C. Fig. 1 represents the variations of the 'increasing degree of inhibition'[2)] when 10% and 0.5% aqueous solutions, respectively,

[1] RC4=0, 0'-p-phenylene-N, N', N'', N'''-tetraethylenetetraimido-diphosphate.

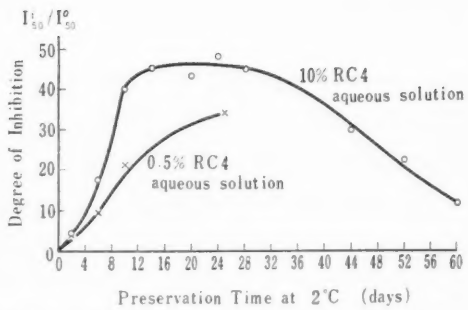

Fig 1. Change of inhibitory activities of RC4 preserved solutions on anaerobic glycolysis of Ehrlich ascites tumor cells. I^t_{50}: 50% inhibitory dose at the time of dissolution; I^o_{50}: 50% inhibitory dose after preservation.

of RC4 were preserved.

Supposing that each reaction product which is to be the cause of the inhibitory activity is identical irrespective of the concentration during preservation, if the rate determining step of the variation is a first order reaction, the formation rate* of the inhibitory substance will be equal irrespective of the concentration during preservation, or if it is a higher order reaction, the formation rate will be elevated as the concentration during preservation is increased.

When the aqueous solutions of RC4, the concentration of which are a and b respectively ($a>b$), are preserved and, their glycolysis inhibiting activities are examined after a certain length of time, let it be assumed that an equal degree of inhibition is observed at the final concentrations (RC4 equivalent) of a' and b' respectively. If the rate determining step is a first order reaction, the formation rate (k) is equal and the concentrations of the inhibitory substance at the time of activity measurement can be represented by ka' and ka' respectively. On the other hand, since the degrees of inhibition of the two solutions are equal, $ka'=ka'$, hence $a'=b'$. This equation indicates that, if the final concentrations are equal at the time of measurement of the inhibitory activity, equal degrees of inhibition are given irrespective of the concentration during preservation, when the rate determining step of the variation is a first order reaction. If the rate determining step is a higher order reaction, the relationship of $a'<b'$ can be given by the same procedure. That is to say, in order to result in equal degrees of inhibition, the final concentration, at the time of activity measurement, of the solution which have been preserved at a higher concentration must be lowered more than that of the solution which have been preserved at a lower concentration, when the rate determining step of the variation is a higher order reaction.

The 50% inhibition was adopted as standard inhibition degree, and the ratios between the 50% inhibitory concentration of the freshly dissolved solution and those of the preserved solutions are used as criterion, instead of directly comparing a' and b'.

* Formation rate:

$$K=\frac{\text{conc. of the inhibitory substance produced during preservation}}{\text{conc. of RC4 aqueous solution preserved}}$$

If c/a'_{50}, equals c/b'_{50}, a first order reaction, or if c/a'_{50} is greater than c/b'_{50}, a higher order reaction must take place, where a'_{50}, b'_{50} are 50% inhibitory concentrations of the preserved solutions, respectively, and c is that of freshly dissolved solution. c/a'_{50} or c/b'_{50} correspond to the 'increasing degree of inhibition' as was designated in the previous report[2]. Namely, by comparing the increasing degrees of inhibition, the reaction order at the rate determining step of the variation can be distinguished.

The 10% aqueous solution gained in its inhibitory activity more rapidly than the 0.5% aqueous solution, which fact suggests participation of a higher order reaction in the activity variation.

On the other hand, the maximum activities of both preserved solutions were almost equal and no heterogeneous difference was found among them on spectral changes in ultraviolet and infrared absorptions, which shall be explained later in this paper. Hence the above stated presumption that the structure of the inhibitory substance is consistent irrespective of the concentration of the preserved solution may be considered reasonable so far.

As was already reported[2], preservation at 37.5°C gaves a similar tendency to the case of preservation at 2°C.

Spectral Variation in Ultraviolet Absorption

The aqueous solution of RC4 was preserved under the same condition as prescribed in the foregoing paragraphs, and was sampled at certain intervals. The ultraviolet absorption spectrum of each sample, after being diluted to 50 μg/ml ($RC_4$4 equivalent), was measured with the Beckman DU-Spectrophotometer equipped with a photomultiplier. The results of preservation of the 10% aqueous solution are shown in Fig. 2. At the time of dissolution, maximum absorption occured at 207.5 mμ and 265 mμ,

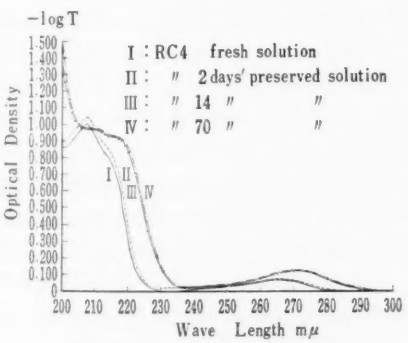

Fig 2 Ultraviolet absorption spectra of 10% RC4 preserved solutions. Each solution was diluted to 50 μg/ml at measuring.

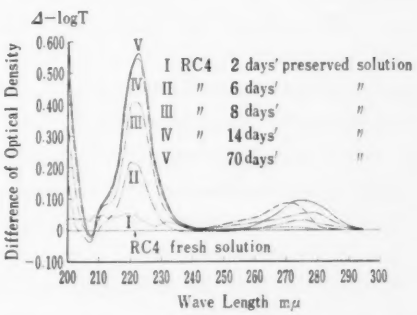
Δ−log T
Difference of Optical Density
I RC4 2 days' preserved solution
II 〃 6 days' 〃
III 〃 8 days' 〃
IV 〃 14 days' 〃
V 70 days' 〃
RC4 fresh solution
Wave Length mμ

Fig. 3 Difference in spectra of preserved RC4 solutions.

but along with the preservation the peak at 207.5 mμ disappeared and shoulders appeared around 210 mμ and 215 mμ instead. Such spectral changes ended in about 14 days of preservation, and after that, even 70 days of preservation hardly made any difference. Fig. 3 represents the difference spectra of the preserved solutions. The freshly dissolved solution was used as the basic line for the measurement. Against this base line the spectra of the preserved solutions were determined.

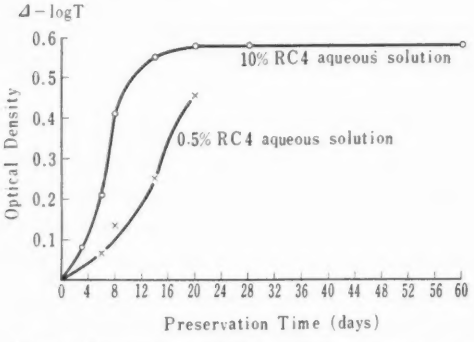

Fig 4. Increase of optical density at 220 mμ of RC4 preserved solutions. Each sample solution was diluted to 50 μg/ml at measuring.

The increase in absorption at 220 mμ is the most conspicuous, and next the variation at 270 to 280 mμ is significant. While preservation of the 0.5% aqueous solution gave an approximately identical change in the spectrum, the rate of change was slower. In Fig. 4, the increase in optical density at 220 mμ, where the increase in absorption was most conspicuous, is plotted against the days of preservation.

Since the spectral change was more rapid with the 10% solution, it is presumed that a higher order reaction takes part in the structural changes of RC4, and this presumption is consistent with the conclusion in regard to Fig. 1.

Spectral Variation in Infrared Absorption The 10% aqueous solution of RC4 was preserved at 2°C, and a portion of it was lyophilized at certain intervals in the course of the preservation. The infrared absorption spectrum of each sample was measured in Nujol mull with the Perkin-Elmer Model 21 Infrared Spectrophotometer.

In order to examine the possibility that the contents of the aqueous solution and those of the lyophilized substance might be different due to progress of a heterogeneous change in the course of lyophilization, the infrared absorption of the lyophilized substance was measured, on one hand, and a portion of it was again dissolved in water and its anti-glycolytic activity and ultraviolet absorption spectrum were measured, on the other. Fig. 5 represents the results of examination on the influence of lyophilization against the glycolysis inhibiting activity. While the activities of the solutions preserved for 2, 4 and 6 days respectively were higher after lyophilization than before the treatment, that of the solution preserved for 22 days showed no difference after lyophilization. Furthermore, when the solution preserved for 1 day was lyophilized and the lyophilized substance was again dissolved in water to give a 10% aqueous solution and was preserved at 2°C, its glycolysis inhibiting

activity increased with the progress of the preservation, and after 7 days reached a point equal to the maximum activity of the preserved solution which had not undergone lyophilization treatment. However, further preservation gave no increase in the activity. Fig. 6 represents the results of examination on the influence of lyophilization on the ultraviolet absorption from the viewpoint of the change in absorption at 220mμ. Those results were quite similar to those shown in Fig. 5.

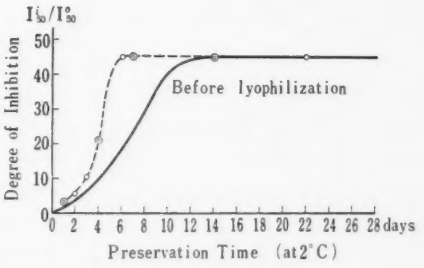

Fig 5. Effect of lyophilization on glycolysis inhibitory activity of RC4 preserved solution. ○: activity after lyophilization; ◎------◎: activity change of redissolved RC4 after lyophilization.

If a heterogeneous change occurred in the course of lyophilization to increase the glycolysis inhibiting activity, same treatment of the 22 day-preserved solution which had already reached its peak of the activity would increase the activity. Or, when the lyophilized substance was again dissolved in water to give a 10% aqueous solution, which was then preserved, the maximum activity achieved by such a solution should be higher than that of the untreated preserved solution by the increment owing to the treatment. However, since such phenomena were not observed, the change during the lyophilization is deemed identical with that in the aqueous solution, the only difference being the accelerated rate of change.

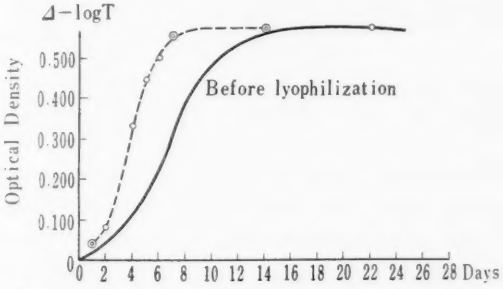

Fig. 6 Effect of lyophilization on absorption change at 220 mμ of preserved RC4 solution. ○: absorption after lyophilization; ◎------◎: absorption change of redissolved RC4 after lyophilization.

After studying the infrared absorption spectra of RC4 and related compounds, Sunagawa and others[3] assigned the key bands of RC4 to be as follows:

ring deformation	1276, 1260, 831, 821, 810 cm^{-1}
P-O-C (aromatic)	1187 cm^{-1}
P=O	1226 cm^{-1}

The representative spectra of lyophilized substances are presented in Fig. 7. The

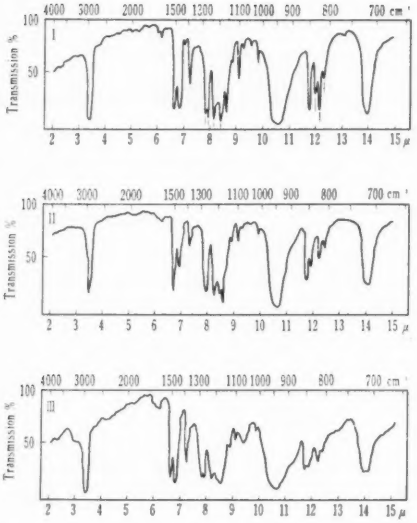

Fig. 7-1 Infrared spectra of RC4 and its lyophilized substances.

I. RC4 crystal
II. RC4 lyophilized after 1 day's preservation
III. RC4 lyophilized after 3 days' preservation

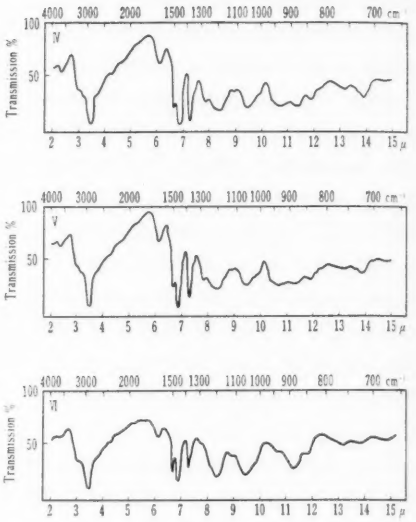

Fig. 7-2 Infrared spectra of lyophilized RC4

IV. RC4 lyophilized after 6 days' preservation
V. 〃 10 days' 〃
VI. 〃 25 days' 〃

ring deformation absorption of ethyleneimino ring decreased with the progress of the preservation, and absorption of 810 to 830 cm^{-1} almost disappeared in the spectrum of a solution preserved for 6 days. On the other hand in the spectrum of a solution preserved for 3 days, absorption occurred at 1064 cm^{-1} presumably due to ether bond and at 3300 cm^{-1} probably due to hydroxyl radical, and these characteristic changes increased with the progress of the preservation. In order to explain these changes, the following hypothetical reaction pathway can be supposed:

$$R\text{-}N\langle^{CH_2}_{CH_2} \xrightarrow{H_2O} RNHCH_2CH_2OH \xrightarrow{R'\text{-}N\langle^{CH_2}_{CH_2}} RNHCH_2CH_2OCH_2CH_2NHR'$$

Since this is a second order reaction, if this reaction is proved to take part in the activity variation, the presumed participation of a higher order reaction in the rate determining step of the variation can be explained without contradiction.

Since pH of the preserved solution was gradually lowered, the appearance of the hydroxyl radicals was partially owing to $O=\overset{|}{\underset{|}{P}}\text{-}OH$. These points will be treated in the subsequent report[4].

In addition, preservation of 0.5% aqueous solution gave spectra substantially identical with those of 10% aqueous preserved solution.

Summary

The correlation between the variation in the glycolysis inhibiting activity and the structural changes when the aqueous solution of RC4 was preserved at 2°C has been examined from the viewpoint of the spectral changes in ultraviolet and infrared absorptions. Participation of a higher order reaction at the rate determining step of the activity variation has been presumed. It is inferred that the ethyleneimino ring is opened along with the progress of the preservation and hydroxyl radical and ether bond newly appear.

Acknowledgments

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References

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4. Maruyama, M. Gann, in press.